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13. ABSTRACT ***(Maximum 200 Words)***

The oxidative stress hypothesis of Parkinson's disease (PD) is thought to involve the superoxide radical and nitric oxide (NO). MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) a meperidine analog, mimics most of the hallmarks of PD and is an important tool that is used to give insights into this debilitating disease. Previous evidence from our laboratory has shown that the superoxide radical is involved in the MPTP neurotoxic process. Herein, we demonstrate a robust gliosis in the substantia nigra of the MPTP-treated mouse which is associated with the marked up-regulation of inducible nitric oxide synthase (iNOS). We also show that mutant mice devoid of the iNOS gene are significantly more resistant to MPTP's toxic effects. These results point to NO as a possible culprit in the MPTP neurotoxic process. In keeping with our hypothesis that NO is part of the oxidative stress hypothesis, we show that MPTP induces, in those brain areas most susceptible to its toxic effects, the formation of 3-nitrotyrosine, o,o-dityrosine and ortho-tyrosine, three stable markers of protein nitration and oxidation. These compounds were also generated *in vitro* by exposing brain proteins to peroxynitrite, a myeloperoxidase-tytosyl radical generation system and a metal-catalyzed oxidation system. These findings are important to our understanding of PD and suggest that inhibition of iNOS may be neuroprotective.

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INTRODUCTION

Parkinson's disease is a common neurodegenerative disorder characterized mainly by tremor, rigidity, akinesia and postural instability (1) all attributed to the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the DA nerve terminals in the caudate-putamen (2,3). To date, the mechanism(s) underlying the death of these neurons in this disorder remains unknown. Symptoms of PD can be alleviated with the use of levodopa, however the progression of the disease still persists (4). Insights into PD have been achieved through the use of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a specific DA neurotoxin that replicates almost all of the hallmarks of PD in non-human primates and in various other mammalian species including a severe irreversible loss of DA neurons in the SNpc and a loss of DA terminals in the caudate nucleus (5-7). Furthermore, mounting evidence such as the production of reactive oxygen species like the superoxide radical and nitric oxide (NO) following MPTP treatment support the oxidative stress hypothesis in the pathogenesis of PD (6). Using transgenic mice that overexpress human superoxide dismutase (SOD), the enzyme responsible for ridding the cell of the superoxide radical, we have demonstrated, previously, that the superoxide radical participates in the MPTP neurotoxic process (7). Three distinct isoforms of nitric oxide synthase (NOS) synthesize NO. Neuronal NOS (nNOS) is the principle NOS isoform in the brain and is constitutively expressed throughout the central nervous system (8-13) whereas endothelial NOS (eNOS) is found mainly in the endothelial layer of blood vessels and in very low concentrations in the brain (14, 15). The third form of NOS, inducible NOS (iNOS), is not expressed at all or is only minimally expressed in the brain (16-18). iNOS expression in the brain has been shown to be increased in pathological conditions such as stroke, AIDS and PD (16-19). In fact, iNOS expression has been demonstrated in the SNpc of post-mortem brain tissues from PD patients (20) indicating that an inflammatory response may be part of the progressive nature of PD. From previous experience, since we and others have found that nNOS knockout mice are only partially protected against MPTP's toxic effects (21-24), we surmised that other NOS isoforms might indeed take part in the MPTP neurotoxic effect. Thus, in experiment 1, using several immunostaining techniques and western blot analyses, we investigated in mouse brain the role of iNOS in the MPTP neurotoxic process. Our findings that iNOS is up-regulated in the SNpc of MPTP-treated mice is complementary to our finding that the superoxide radical is involved in the death of DA neurons following MPTP treatment. Since the superoxide radical and NO are weak oxidants, individually neither is sufficiently damaging. However, reaction between the two can produce peroxynitrite, a reactive nitrogen species that can oxidixe the phenolic rings, particularly tyrosine, of proteins and damage DNA (25,26). Markers for the oxidation of tyrosine, nitrotyrosine and dityrosine, are indicative of the extent of peroxynitrite-induced damage to tyrosine residues which are so important to phosphorylation, signal transduction and catecholamine synthesis in the cell (27). Thus, in our continuing studies to elucidate the mechanism of cell death in the MPTP neurotoxic process, using isotope dilution gas chromatography-mass spectrometry, we determined levels of oxidized and nitrated tyrosine residues and oxidized proteins in mouse brain tissues following MPTP administration.

BODY OF RESEARCH

Our overall long-term goal is the study of the pathogenesis of PD. To accomplish this, we have put forth a series of studies using the specific DA neurotoxin MPTP that hopefully will afford us more insights into the cause(s) of this debilitating disease and possible therapies thereof. Previously, we have provided strong support for the oxidative stress hypothesis of PD. Data from our MPTP studies demonstrate that both the superoxide radical and NO can react with each other to produce the strong oxidant peroxynitrite which can damage proteins and DNA.

Specific Aim I

To further understand the roles of the superoxide radical and NO in the MPTP neurotoxic process, **Specific Aim I** of this award proposes to determine the contributions of superoxide, NO or both to MPTP neurotoxicity by administering this toxin to different lines of mice that are genetically engineered to exhibit a greater capacity for detoxifying superoxide (transgenic copper/zinc-superoxide dismutase [SOD1] mice) and/or a lower capacity for synthesizing NO (knockout neuronal NO synthase [nNOS] mice and by assessing the status of the nigrostriatal DA pathway in these different types of mice following MPTP administration using high performance liquid chromatography (HPLC) and immunostaining with quantitative morphology. We have not started work on **Specific Aim I** yet as it is necessary to breed and crossbreed these mice for this experiment. Prior to the breeding of these mice for this part of our studies, it is necessary to put them on a C57/BL mouse background as this is the mouse background that we have used for our original MPTP studies.

Specific Aim II

As stated above, there are three isoforms of the NOS enzyme. Therefore, the question is which NOS isoform is the main contributor to NO formation in the MPTP mouse model of PD and in PD itself? Previous data from our laboratory demonstrates that the nNOS inhibitor, 7-nitroindazole at doses that inhibit about 80% of nNOS activity, completely protected against MPTP-induced DA toxicity in mice whereas nNOS knockout mice, which have about 10% residual nNOS activity, were only partially protected against MPTP-induced DA toxicity. These results indicate that although nNOS may be important in the MPTP-induced DA neurotoxic process, it may not be the only NOS isoform involved in this process. Of note is the fact that considerable iNOS expression has been found in the SNpc of post-mortem tissues from PD patients (20). It has also been found in other situations where neurodegeneration occurs such as in Alzeheimer's disease and stroke (16-19), thus, the speculation that an inflammaroty (microglia involvement) situation may be part of the neurodegenerative process. Since iNOS is only minimally or not expressed in the brain under normal conditions and up-regulated following MPTP administration, **Specific Aim II** addressed the question of the source of NO in the SNpc following MPTP administration to mice. For a detailed description of the materials and methods for Specific Aim II, see enclosed Liberatore et al., 1999.

Results

MPTP produces a Robust Glial Response.

The macrophage antigen-1 (MAC-1) and glial fibillary acidic protein (GFAP), specific markers for microglia and astrocytes were used to gauge the glial response in the SNpc of MPTP-treated C57/BL mice. In saline-treated animals, faintly immunostained microglia and astrocytes indicate the resting state of the microglia (figure 1a-c). In the MPTP-treated ventral midbrain, glial immunoreactivity are significantly greater than in control, span the entire SN and show even stronger reactions in the SNpc as demonstrated in figure 1a-c. Alterations in MAC-1 indicating activated microglia were evident as early as 12 hours after the last dose of MPTP, persisted to its peak between 24 and 48 hours and was no different from control by 7 days after acute MPTP (figure 1). On the other hand, GFAP (astrocytic) alterations were noticeable at 24 hours, maxed out at 4-7 days and remained above control even at 21 days (figure 1d-f). Striatal responses were similar to those of the ventral midbrain (data not shown).

MPTP stimulates iNOS expression in glial cells.

The number of iNOS-positive cells in the SNpc of the saline-mice were rare to non-existent (figure 2). However, in MPTP-treated mice, iNOS-positive cell numbers in the SNpc increased to 250% over control by 24 hours and had returned to control levels by 48 hours (figure 2). To determine the nature of these iNOS-positive cells in the SNpc of MPTP-treated mice, we used a double staining procedure in which we immunostained simultaneously for iNOS and either MAC-1 or GFAP. Twenty-four hours after the last dose of MPTP at the time when iNOS-positive cells reach their peak in the SNpc, MAC-1-positive activated microglia showed iNOS-positive immunoreactivity (figure 2). GFAP-positive cells did not exhibit any iNOS immunostaining and no iNOS-positive cells were found in the striatum at any time point following MPTP.

iNOS mRNA levels and enzymatic activity increase following MPTP.

Ventral midbrain of saline-injected mice show very little mRNA (figure 3). In contrast, iNOS mRNA levels in midbrain from MPTP-injected mice were detected as early as 12 hours, reached maximum levels at 48 hours and was undetectable by 4 days after MPTP injection (figure 3). Striatal iNOS mRNA levels remained low throughout the entire time-course study. In agreement with ventral midbrain mRNA levels, iNOS enzymatic activity in saline injected mice very low whereas iNOS enzymatic activity in this brain area increased rapidly as early as 12 hours following MPTP. INOS enzymatic activity peaked at 48 hours then slowly receded to control activity by 7 days. Like striatal mRNA levels, striatal iNOS enzymatic activity was not affected by MPTP throughout the entire time course study. nNOS enzymatic activity was consistently higher than iNOS enzymatic activity and was unchanged following MPTP administration.

iNOS-deficient mice are resistant to MPTP neurotoxicity.

Since MPTP increases iNOS expression and up-regulates iNOS mRNA levels in normal wild-type mice, the definitive proof that iNOS is the primary sourse of NO in the MPTP neurotoxic process is to examine MPTP's neurotoxic effects in mice deficient in the iNOS enzyme. iNOS knockout mice ($iNOS^{-/-}$) received the same MPTP dosing schedule

or saline as wild-type littermate mice and were sacrificed at the same time points. Stereological counts of SNpc DA neurons, defined by tyrosine hydroxylase (TH) and Nissl staining, revealed no differences in cell count numbers between saline-injected $iNOS^{-/-}$ mice and their wild-type littermates (Table 1). In the MPTP-treated groups, 29% of the TH-immunostained and 46% of the Nissl-stained SNpc neurons in wild-type mice survived MPTP whereas twice as many TH-immunostained and Nissl-stained neurons in the $iNOS^{-/-}$ group survived the MPTP onslaught. Interestingly, the extent of loss of striatal DA, DOPAC and HVA was not different between both groups of mice after MPTP treatment (Table 2).

Microglial responses and MPP+ $iNOS^{-/-}$ mice.

$iNOS^{-/-}$ mice exhibit the same increases in MAC-1 immunostaining as their wild-type littermates following MPTP treatment even though they possess no $iNOS^{-/-}$ gene (figure 5). However, they also exhibit no differences from their wild-type littermates in striatal MPP+ accumulation following MPTP administration (Table 3).

Brain nitrotyrosine levels in $iNOS^{-/-}$ and wild-type littermate mice.

NO is reported to damage DNA and nitrate the tyrosine residues in the phenolic rings of proteins. Using dot-blot analysis, we determined nitrotyrosine (NT) levels in selected brain regions of $iNOS^{-/-}$ mice and their wild-type littermates following either saline or MPTP injections. In the saline-injected mice, striatum and cerebellum had the highest NT levels followed by cerebellum and frontal cortex (Table 4). In the MPTP-injected mice, NT levels were significantly increased in striatum and ventral midbrain but unchanged in cerebellum and frontal cortex (Table 4). However, NT increases in ventral midbrain from $iNOS^{-/-}$ mice were smaller than those in their wild-type littermates. On the other hand, NT increases in striatum from $iNOS^{-/-}$ mice and their wild-type littermates were similar (Table 4)

Discussion

The present study demonstrates that gliosis is a prominent neuropathological response in the SNpc and the striatum in the MPTP mouse model as in PD (28,29). Our data suggest that inflammatory-related events, such as gliosis, may be a part of the degeneration of dopaminergic neurons in the MPTP model. For example, activated microglial cells in the SNpc appear much earlier than reactive astrocytes when only minimal neuronal death has occurred (30). This supports the view that because the microglial response to MPTP occurs early enough in the neurodegenerative process, it may contribute to the death of SNpc dopaminergic neurons. We also found that these cells increase in number following MPTP administration, and that they are the site of iNOS upregulation. Thus, it may be that activated microglial cells can overwhelm surrounding dopaminergic neurons with significant amounts of iNOS-derived NO as well as with other reactive species such as superoxide radicals (31) which is in keeping with the deleterious role of microglia (31). Astrocytic responses to MPTP were quite distinct from that of microglia since changes in the density of reactive astrocytes in both striatum and SNpc paralleled rather than preceded the active phase of dopaminergic neuron degeneration (30). Therefore, the observed astrocytic reaction is not a primary event in the loss of dopaminergic neurons

which suggests that reactive astrocytes may propogate rather than initiate the neurodegenerative process.

Experiments, both *in vitro* and *in vivo,* indicate that iNOS transcription can be induced by cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) (32-34) as well as by ligation of the macrophage cell surface antigen CD-23 (35). Relevant to PD, is the fact that glial cells immunoreactive for TNF-α, IL-1β, IFN-γ, and CD-23 has been detected in the SNpc of PD brains (36). In agreement with this transcriptional induction model of iNOS, we found iNOS mRNA levels increased in the ventral midbrain of MPTP-injected mice but not in the striatum of these mice even in the face of a strong glial reaction here. Among various possibilities, it may be hypothesized that the discrepancy in the iNOS response between striatum and ventral midbrain reflects either different modes of iNOS regulation in these two brain regions or the existence of a striatal factor that suppresses the induction of iNOS (37).

Our data on microglial iNOS immunoreactivity in the SNpc of MPTP-injected mice agree with the situation reported in PD brains in which iNOS immunoreactivity was observed in SNpc microglia/macrophages (20). It also provides critical insights into these autopsy findings, by suggesting that iNOS upregulation is not due to the chronic use of anti-PD drugs such as L-DOPA, nor to any alteration that occurs at end-stage PD. Our findings do, however, feed into the hypothesis that iNOS-mediated NO and superoxide production (31) may contribute to the neurodegenerative process in this model and in PD. Since NO can traverse membranes and diffuse to neighboring neurons, whereas superoxide cannot readily transverse cellular membranes (38), it is unlikely that microglial-derived extracellular superoxide has access to dopaminergic neurons to directly trigger intracellular toxic events. But, NO could react with superoxide in the extracellular space to form the highly reactive tissue-damaging species, peroxynitrite, which can cross the cell membrane and injure neurons. Therefore, microglial-derived superoxide, by contributing to peroxynitrite formation, may be significant in this model. The presumed absence of a direct role for extracellular superoxide in the MPTP-induced neurotoxic process does not negate the previously demonstrated instrumental role of intracellular superoxide in this MPTP model (6), especially that produced within dopaminergic neurons consequent to the mitochondrial electron transport chain blockade by MPP^+.

Twice as many SNpc dopaminergic neurons survived MPTP administration in *iNOS*$^{-/-}$ mice compared to their wild-type littermates which is consistent with the involvement of iNOS in the MPTP neurotoxic process. Since activated microglia can exert deleterious effects unrelated to NO, it must be emphasized that the *iNOS*$^{-/-}$ mice showed no evidence of impaired microglial activation in response to MPTP and that *iNOS*$^{-/-}$ macrophages, which do not produce NO, remain responsive to IFN-γ and, once activated, preserve their respiratory burst capacity (39) that includes the formation of superoxide (40). We also found that ablation of iNOS was not associated with alterations in the formation of the MPTP active metabolite, MPP^+, which is the most significant modulating factor of MPTP potency (40). Thus, it can be postulated that the resistance of *iNOS*$^{-/-}$ mice to MPTP is due to the lack of iNOS expression and reduced NO formation, and not to a microglial-deficient respiratory burst capacity or an altered MPTP metabolism. The resistance of the SNpc dopaminergic neurons in *iNOS*$^{-/-}$ mice was not accompanied by a comparable sparing of striatal dopaminergic fibers, as levels of dopamine and its metabolites following MPTP administration were similarly decreased in *iNOS*$^{-/-}$ mice and their wild-

type littermates. In the SNpc, MPTP caused significant iNOS upregulation and, constitutively, there are only a few midbrain nNOS-positive neuronal elements which are not of dopaminergic nature and which do not have a close relationship with SNpc dopaminergic neurons (9, 41). Conversely, in the striatum, MPTP did not cause any detectable iNOS upregulation and, constitutively, there are numerous nNOS-positive neurons and nerve fibers (9). Therefore, the significant preservation of the SNpc dopaminergic neurons with the loss of striatal dopaminergic fibers in MPTP-injected *iNOS*$^{-/-}$ mice can be explained by the fact that MPTP-mediated damage of striatal dopaminergic nerve fibers, unlike SNpc dopaminergic neurons, do not significantly rely on NO produced by iNOS, but rather by other NOS isoforms such as nNOS (22).

We noted that ventral midbrain iNOS enzymatic activity was about six-fold lower than nNOS enzymatic activity in this same brain region. Thus, how could iNOS, a seemingly insignificant component of total NOS enzymatic activity, significantly modulate MPTP-induced SNpc injury. Since NO must travel from the its site of release to the dopaminergic neurons and ventral midbrain nNOS-positive neuronal elements are away from dopaminergic structures, it is likely that the amount of nNOS-derived NO that reaches the target neurons is much less than one could anticipate in light of nNOS catalytic activity. On the other hand, ventral midbrain iNOS-positive microglial cells are in close proximity to dopaminergic structures which would make the amount of iNOS-derived NO reaching target neurons conceivably quite significant. Furthermore, since nNOS activity is under the dynamic regulation of calcium, it is probable that midbrain nNOS does not produce anywhere near six-fold as much NO as iNOS.

From a mechanistic point of view, NO is a weak oxidant, thus, by itself, it is not sufficiently damaging enough to participate directly in MPTP's deleterious effects. However, NO-derived species with stronger oxidant properties, such as peroxynitrite, can indeed cause direct injury to the dopaminergic neurons (42). Peroxynitrite can also nitrate tyrosine residues (43) which may serve as a stable biomarker for peroxynitrite actions. Our demonstration that nitrotyrosine levels were significantly increased following MPTP administration only in brain regions known to be susceptible to the toxin support the involvement of peroxynitrite in MPTP neurotoxic process (21, 24, 25). More striking still is the fact that the MPTP-injected *iNOS*$^{-/-}$ mice, which showed significantly less SNpc dopaminergic neuronal loss, also showed significantly smaller increases in ventral midbrain nitrotyrosine levels compared to their wild-type counterparts.

Our data provide clear evidence for a pivotal role of microglial iNOS-derived NO in the cascade of deleterious events that ultimately leads to SNpc dopaminergic neuronal death in the MPTP mouse model and in PD and suggest that the inhibition of iNOS may be a valuable target for the development of new therapies for PD aimed at attenuating the actual loss of dopaminergic neurons. However, since this study also shows that iNOS inhibition is not effective in preserving striatal nerve fibers from MPTP neurotoxicity, the ideal therapeutic approach for PD may rely on the combination of iNOS inhibitors with agents that promote nerve fiber re-growth, stimulate dopaminergic function and preserve dopaminergic nerve terminals. This makes perfect sense as multi-drug strategies have been successful in the treatment of other pathological conditions, such as HIV infection and cancer.

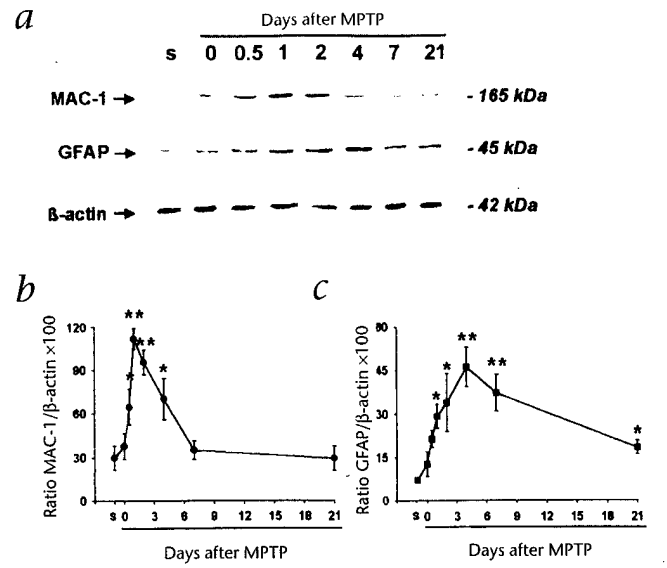
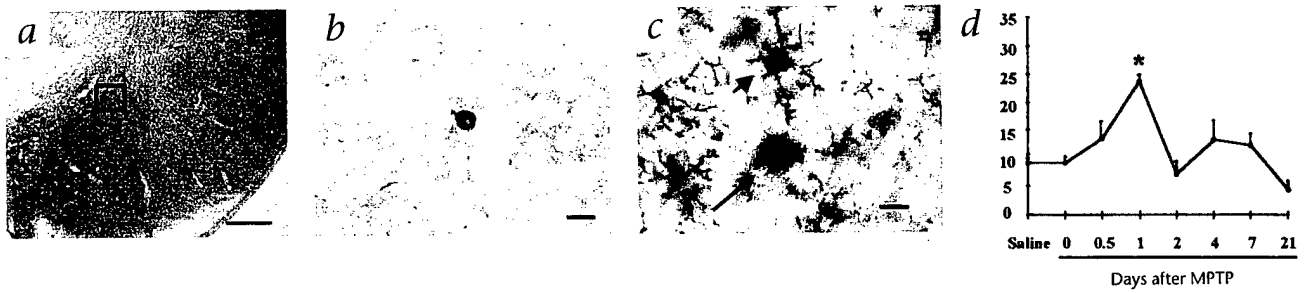


Fig. 1 MPTP-induced glial reaction. ***a–c***, Ventral midbrain MAC-1 (*a* and *b*) and GFAP (*a* and *c*) expression is minimal in saline-injected mice (S), but increases in a time-dependent manner after MPTP injection. Data represent mean ± s.e.m. ($n = 4–5$). **, $P < 0.01$ and *, $P < 0.05$, compared with saline, Newmas-Keuls post-hoc test. ***d–i***, There is a robust MAC-1 (*d*) and GFAP (*g*) immunostaining in the SNpc of MPTP-treated mice compared with that in saline-treated control mice (*f* and *i*) at 24 h after injection. *e* and *h*, Magnification of the boxed areas in *d* and *g* shows that the MAC-1- and GFAP-immunoreactive cells in the MPTP-treated mice seem to have a morphology typical of activated microglia cells (*e*) and of reactive astrocytes (*h*). Scale bars represent 200 µm (*d,f,g,l*; shown in *d*) and 15 µm (*e,h*; shown in *e*).



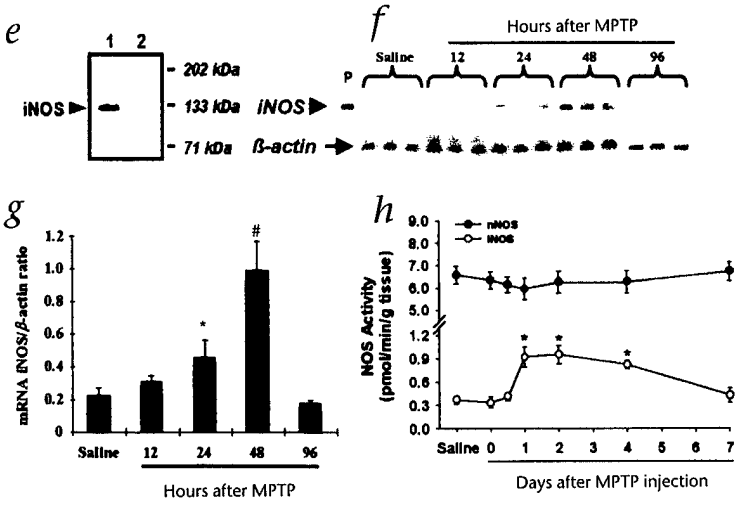


Fig. 2 MPTP-induced iNOS upregulation. ***a*** and ***b***, SNpc of a mouse 24 h after MPTP injection (*a*), containing an iNOS-positive cell (*b*; enlargement of boxed area in *a*). ***c***, All iNOS-positive cells (black) co-localize with MAC-1 immunoreactivity (violet) (long arrow), but several MAC-1-positive cells do not co-localize with iNOS immunoreactivity (short arrow). Scale bars represent 200 µm (*a*) and 10 µm (*b,c*). ***d***, Time course of iNOS-positive cell numbers in the SNpc after MPTP injection ($n = 4–5$ mice per time point). *, $P < 0.05$, compared with all other groups, Newmas-Keuls post-hoc test. ***e***, Western blot analysis of protein extract from lipopolysaccharide-treated mouse cells demonstrates that the antibody against iNOS used here recognizes a single band with an apparent molecular mass of 130 kDa (lane 1), which is consistent with iNOS; when the antibody against iNOS is omitted the band is not seen (lane 2). Right margin, molecular sizes. ***f*** and ***g***, Ventral midbrain iNOS mRNA levels are increased by 24 and 48 h after MPTP injection compared with those of saline-injected mice, but return to basal levels by 96 h. Data are from three mice per group and are representative of at least three independent experiments. P, iNOS-positive control (lipopolysaccharide-treated mouse cells). *, $P < 0.05$ and #, $P < 0.01$, higher than all other groups, Newmas-Keuls post-hoc test. ***h***, Ventral midbrain iNOS activity is significantly increased 1 d after MPTP injection, is still increased at 4 d although it returns to basal levels by 7 d after MPTP injection, whereas ventral midbrain nNOS activity remains unchanged throughout. *$P < 0.01$, higher than all other groups, Newmas-Keuls post-hoc test.

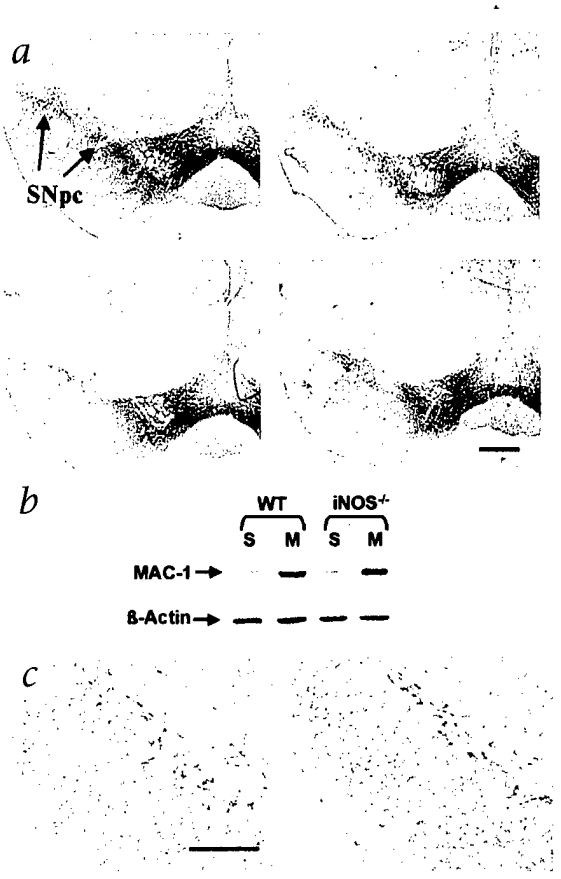


Fig. 3 MPTP-induced neuronal loss and microglial reaction in *iNOS*$^{-/-}$ mice. ***a***, SNpc TH-positive neurons are twice as resistant to MPTP in *iNOS*$^{-/-}$ mice than in wild-type (WT) littermates, 7 d after MPTP injection. In addition, there is no noticeable difference in TH-positive neuron density after saline injection between *iNOS*$^{-/-}$ mice and WT littermates (Table 1, actual neuronal counts). ***b*** and ***c***, Ventral midbrain western blot analysis (*b*) and SNpc immunohistochemical analysis (*c*) for MAC-1, showing the similarity of the microglial response between the *iNOS*$^{-/-}$ mice and their wild-type littermates (WT), 24 h after MPTP injection. Scale bar represents 200 μm (*a* and *c*, shown in *c*).

Table 1 Number of neurons in the SNpc

	Saline		MPTP	
	Wild-type	*iNOS*$^{-/-}$	Wild-type	*iNOS*$^{-/-}$
Tyrosine hydroxylase	10,188 ± 619	9,680 ± 496	2,940 ± 698*	5,720 ± 539**
Nissl	13,563 ± 1007	12,913 ± 530	6,300 ± 539*	10,417 ± 380**

SNpc neurons (mean ± s.e.m.; $n = 5$ per group) were counted by stereology. *, $P < 0.001$, fewer than both saline-injected groups; **, $P < 0.05$, fewer than both saline-injected groups and more than MPTP-injected wild-type mice; Newman-Keuls post-hoc test.

Table 2 Striatal monoamine levels (ng/mg tissue)

	Dopamine	DOPAC	HVA
Saline ($n = 6$)	14.7 ± 0.8	1.6 ± 0.3	2.9 ± 0.1
MPTP			
Wild-type ($n = 4$)	2.8 ± 0.5*	0.7 ± 0.2*	1.8 ± 0.2*
iNOS$^{-/-}$ ($n = 5$)	2.4 ± 0.3**	0.5 ± 0.1**	1.7 ± 0.2**

As dopamine, DOPAC, and HVA values did not differ between saline-injected *iNOS*$^{-/-}$ and their saline-injected wild-type littermates, data from both groups were combined. *, $P < 0.01$, different from saline-injected control mice but not MPTP-injected *iNOS*$^{-/-}$ mice; **, $P < 0.01$, different from saline-injected control mice but not MPTP-injected wild-type mice; Newman-Keuls post-hoc test. Data represent means ± s.e.m. for four to six mice per group.

Table 3 MPP^+ levels (µg/g striatum)

	90 min	120 min	180 min	360 min
Wild type	17.75 ± 0.50	23.10 ± 4.30	19.17 ± 0.63	1.61 ± 0.12
iNOS$^{-/-}$	24.24 ± 2.70	20.61 ± 2.23	17.18 ± 0.83	1.91 ± 0.49

Striatal MPP^+ levels in wild-type and *iNOS*$^{-/-}$ mice at 90, 120, 180 and 360 min after the last MPTP injection do not differ ($P > 0.05$; Newman-Keuls post-hoc test) between the two groups. Data represent means ± s.e.m. for four mice per group and time point.

Table 4 Nitrotyrosine levels (ng/µg protein)

	Frontal Cortex	Striatum	Ventral Midbrain	Cerebellum
Wild-type				
Saline	18.9 ± 3.7	30.9 ± 2.1	13.3 ± 3.2	21.6 ± 3.2
MPTP	19.1 ± 2.1	67.8 ± 3.1*	31.7 ± 1.7*	23.5 ± 2.2
iNOS$^{-/-}$				
Saline	18.4 ± 3.5	28.3 ± 2.5	14.0 ± 2.5	20.6 ± 1.8
MPTP	19.6 ± 2.5	68.4 ± 2.9**	21.9 ± 1.7***	19.1 ± 2.0

*, $P < 0.01$, higher than saline-injected mice; **, $P < 0.01$, higher than saline-injected mice but not MPTP-injected wild-type mice; ***, $P < 0.05$, higher than saline-injected mice but lower than MPTP-injected wild-type mice; Newman-Keuls post-hoc test. Data represent mean ± s.e.m. for three to four mice per group and treatment.

Key Research Accomplishments in the Specific Aim II Study.

Our accomplishments in Specific Aim II have indeed added one more piece in our understanding of the puzzle of PD. Using the best tool available for the study of PD, MPTP, we have confirmed some of the findings that already exist for post-mortem SNpc tissues and we have also added some new findings. Listed below are the accomplishments in Specific Aim II which has been completed according to the Statement of Work. These findings have been published in Nature Medicine 5: 1403-1409 (1999).

- MPTP produces a strong glial response in SNpc of mice which is in agreement with what has been found in the post-mortem SNpc tissues from PD brains.
- MPTP stimulates iNOS expression in microglial cells in the SNpc of treated mice. This finding is in agreement with reports of finding iNOS immunoreactivity in microglia in SNpc from post-mortem PD brains.
- MPTP increases iNOS mRNA levels and enzymatic activity in selected brain regions from treated mice which indicates that iNOS plays a significant role in the MPTP-induced neurodegeneration in the SNpc and suggests that this may be the case in PD.
- iNOS-deficient mice are more resistant to MPTP than their wild-type littermates but accumulate MPP+ similar to their wild-type littermates. They are also not resistant to the loss of DA and its metabolites.

References for Specific Aim II

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Specific Aim III.

The reaction between the superoxide radical and NO has been proposed to produce the even stronger oxidant peroxynitrite which can damage DNA and nitrate tyrosine residues in proteins (1). This reaction may be relevant to the damage seen in SNpc DA neurons. Markers of oxidative damage to DNA and nitration of tyrosine residues, dityrosine and nitrotyrosine, have been identified. In a previous experiment (2), we have established that MPTP stimulates significant amounts of superoxide radicals. And, recently, we have documented that iNOS is most likely the major source of NO in the MPTP mouse model of PD (see ***Specific Aim II***). Increased iNOS expression in the SNpc represents an inflammatory response to some sort of injury and based on our findings, the culprit causing this injury may indeed be peroxynitrite. It follows then that in furthering our understanding of PD, the measurement of markers for DNA damage and nitration of tyrosine residues following MPTP administration is necessary. Thus, in **Specific Aim III**, using gas chromatography with mass spectrometry, we measured dityrosine and nitrotyrosine in selected brain regions and at selected time points from wild-type mice that were treated with MPTP. The methods and results of this work have been published in the Journal of Biological Chemistry (Pennathur et al., J Biol. Chem. 274: 34621-34628, 1999, see enclosed publication). Since the basic part of this work has been completed, we will now begin to measure 3-nitrotyrosine, o,o-dityrosine and ortho-tyrosine in transgenic human SOD1, nNOS and iNOS mice.

Results

Mass Spectrometric detection of 3-Nitrotyrosine, o,o-dityrosine and ortho-tyrosine in mouse brain.

Oxidized amino acids were analyzed in freshly isolated tissues from ventral midbrain, striatum, cerebellum and frontal cortex using acid hydrolysis followed by derivization with *n*-propanol and heptafluorobutyric anhydride the put through GC/MS analysis in the negative-ion electron capture mode. Compounds were detected that exhibited major ions and retention times that were identical to authentic 3-nitrotyrosine (3-NT), ortho-tyrosine and o,o-dityrosine (figure 1). Ion monitoring demonstrated that the ions derived from the amino acidsco-eluted with ions derived from authentic ^{13}C-labeled internal standards. In normal mouse brain, levels of oxidized amino acids varied in magnitude in different parts of the mouse brain (figure 1). All regions had relatively high amounts of ortho-tyrosine, highest amounts being found in the striatum. Levels of 3-NT and o,o-dityrosine were lower than those of ortho-tyrosine and o,o-dityrosine levels were lowest in the striatum. 3-NT levels were comparable in all regions studied

3-Nitrotyrosine is elevated in ventral midbrain and striatum of MPTP-treated mice.
To determine whether MPTP promotes oxidative damage to brain proteins, ventral midbrain, striatum and two brain regions supposedly not affected by MPTP, cerebellum and frontal cortex from saline-injected and MPTP-treated mice were analyzed for 3-NT levels. Levels of 3-NT in ventral midbrain (+110%) and striatum (+90%) were markedly elevated following MPTP administration as compared to saline-injected mice (figure 2). Results indicated that the observed increases were selective for regions of the brain that were susceptible to the neurotoxic effect of MPTP.

o,o-Dityrosine is elevated in ventral midbrain and striatum of MPTP-treated mice.
Elevated levels of o,o-dityrosine in ventral midbrain (120%) and striatum (170%) following MPTP administration were strikingly similar to those of 3-NT following MPTP treatment as compared to control levels (figure 3). Both regions are susceptible to the toxic effects of MPTP. As with 3-NT, cerebellum and frontal cortex exhibited no level changes. Furthermore, results indicate that like 3-NT o,o-dityrosine level increases occur only in those brain regions that are susceptible MPTP's toxic effect.

Ortho-Tyrosine levels are unchanged in all brain regions in the MPTP-treated mouse.
In contrast to the changes observed in 3-NT levels and in o,o-dityrosine levels in ventral midbrain and striatum of MPTP-treated mice, there were no changes observed in the levels of ortho-tyrosine in any of the brain regions from MPTP-treated mice that were analyzed for this compound (figure 4)

3-Nitrotyrosine, o,o-Dityrosine and Ortho-tytosine in Brain Tissues Oxidized by Peroxynitrite *in Vitro*.
3-Nitrotyrosine, o,o-dityrosine anprotein d ortho-tytosine seem to be markers of damage to proteins and amino acids. Thus, to evaluate the usefulness of these compounds as markers for oxidative damage, product yields of these compounds in brain proteins were investigated following *in vitro* exposure of brain tissues to peroxynitrite, tyrosyl radical and the hydroxyl radical ($HO^{\cdot}$). The proteins used for the *in vitro* exposure study came from homogenates prepared from ventral midbrain, striatum, cerebellum and frontal cortex. In brain proteins exposed to peroxynitrite, there was a significant increase (80 fold) in 3-NT in all brain proteins following peroxynitrite exposure and proteins from different regions all demonstrated similar increases (figure 5A). Interestingly, when protein homogenates were exposed to peroxynitrite after 2 minutes, protein nitration was minimal which indicated that peroxynitrite is a short-lived species and that it does nitrate the tyrosine residues in proteins. Prior studies have identified 3-NT as a specific markerfor protein oxidation by reactive nitrogen species. Ortho-tyrosine and o,o-dityrosine levels increased 2-3 fold in brain proteins after peroxynitrite exposure, however product yields were <5% and <25% respectively (figures 5B and 5C).

3-Nitrotyrosine, o,o-Dityrosine and Ortho-tyrosine in Brain Protein Oxidized *in Vitro* by Myeloperoxidase-Generated Tyrosyl Radical.
Having generated the tyrosyl radical using an *in vitro* myeloperoxidase-tyrosine-H2O2 generation system, proteins from different areas of the brain were exposed to this tyrosyl

radical. The major product of this reaction was o,o-dityrosine and all homogenates from the different brain regions exposed to the tyrosyl radical exhibited similar increases in this compound (figure 6). There were no changes in levels of 3-NT or ortho-tyrosine. Recent evidence showed that the myeloperoxidase-H2O2 system will convert tyrosine into 3-NT in a reaction that requires nitrite, a degradation product of NO. Brain proteins from specific brain regions exposed to this myeloperoxidase-H2O2 system with the addition of nitrite showed 3-NT levels similar to those observed in the MPTP mouse model of PD. Results here indicate an alternate pathway for the formation of o,o-dityrosine and 3-NT, this one involving the tyrosyl radical while the other pathway involves reactive nitrogen species.

3-Nitrotyrosine, o,o-Dityrosine and Ortho-tyrosine in Brain Proteins Oxidized by the Hydroxyl Radical in Vitro.

Proteins isolated from cerebellum, frontal cortex, ventral midbrain and striatum of control mice were exposed to a $HO^{\cdot}$ generating system which contains copper and H2O2. This system generates the $HO^{\cdot}$ and other reactive species as well. Levels of 3-NT, o,o-dityrosine and ortho-tyrosine were determined in hydrolysates of the proteins following exposure using GC/MS. Ortho-tyrosine and o,o-dityrosine levels were detected in proteins from all four brain regions (figure 7A and 7B). Levels of ortho-tyrosine were 10 fold higher than that of o,o-dityrosine. In contrast, the levels of 3-NT remained unchanged. These same results have been observed in model proteins following exposure to ionizing radiation. These results suggest that ortho-tyrosine might serve as a marker for protein damage resulting from $HO^{\cdot}$ exposure.

Discussion

The present studies investigated the pathways that oxidatively damage proteins in the brain under normal and pathological conditions using highly sensitive and specific isotope dilution mass spectrometric methods. The focus of these studies was on three oxidants that have potential pathological significance: $ONOO^{-}$, the product of the reaction between NO and the superoxide radical; the tyrosyl radical,generated by the heme protein myeloperoxidase; and $HO^{\cdot}$ radical generated by a copper-catalyzed oxidation system. As markers of protein oxidation, we chose 3NT, o,o-dityrosine and ortho-tyrosine because they are stable to acid hydrolysis and because they probably represent post-translational modifications of proteins. In normal mice, detectable levels of 3-NT, o,o-dityrosine and ortho-tyrosine were found in all brain regions studied. This finding is in agreement with the view that basal levels of oxidatively damaged proteins exist under normal physiological conditions in the brain and probably relates to its high level of oxygen consumption and less-than-satisfactory antioxidant protection system. Proteins and DNA as well are oxidized under "normally stressful" situations and significant levels of 8-hydroxy-deoxyguanosine, a marker for DNA oxidative damage, are found in rodent and human brains (10,11). Oxidative stress may be normal here and may eventually lead to what we call the aging process in the brain which is characterized by the decline of physiological functions over time (12). Levels of the observed markers varied among the different brain regions. For example, 3-NT and ortho-tyrosine levels differed in the various brain regions with highest levels in the striatum. Of note, is the fact that the striatum is among those brain regions that showed the highest levels of the DNA damage

marker 8-hydroxy-deoxyguanosine. These findings support the belief that the basal ganglia are more vulnerable to oxidative attack and is more often affected in oxidant-related neurodegenerative disorders (1).

Brains from the MPTP-treated mice exhibited strikingly elevated levels of both 3-NT and o,o-dityrosine compared to saline-treated mice. Significant increases were found in striatum and ventral midbrain but not in cerebellum and frontal cortex. This is consistent with MPTP's neurotoxic effects on the nigrostriatal DA pathway and indicate that MPTP promotes protein oxidation especially in brain regions where its effect is known to be severe. Evidence is also provided that MPTP stimulates both the production of reactive species and oxidative damage to elements critical to cellular function. When brain proteins were exposed *in vitro* to $HO^{\cdot}$ generated by a metal-catalyzed oxidation system, ortho-tyrosine was the major oxidation product. However, in the MPTP model, ortho-tyrosine was not altered in the brain tissues. MPTP has been proposed to enhance $HO^{\cdot}$ production by impairing mitochondrial respiration (3,13,14). But the lack of any detectable increase in ortho-tyrosine in the brains of MPTP-treated mice argues against a serious role for $HO^{\cdot}$ in protein damage and doubts the participation of this oxy-radical in MPTP-induced toxicity *in vivo*.

The marked increase in 3-NT levels following MPTP administration strongly suggests that reactive nitrogen intermediates play a key role in protein oxidation. Compelling evidence for this view comes from our *in vitro* experiments with $ONOO^{-}$ in which we show that $ONOO^{-}$ causes a striking elevation of 3-NT with a significant increase in ortho-tyrosine and a much smaller increase in o,o-dityrosine. Our results are in agreement with earlier demonstrations that inhibition of NOS attenuates MPTP-induced DA toxicity (2-4). Beal et al. (17) showed that MPTP significantly increases striatal levels of free 3-NT in mice. The relationship between free 3-NT and 3-NT in proteins is unknown and the pathophysiological significance of free 3-NT remains to be determined. Protein nitration may be deleterious as it alters the pka of tyrosine and can affect the protein's secondary and tertiary orientation and organization which can alter its function as we have shown for tyrosine hydroxylase (7). Thus, not only do we confirm that MPTP increases 3-NT levels in the brain, we also provide support to the hypothesis that tyrosine nitration of brain proteins may play a critical role in the MPTP neurotoxic process.

Increases in o,o-dityrosine following MPTP administration to mice suggest the involvement of a second physiologically relevant pathway for producing protein oxidation that involves the tyrosyl radical and heme proteins. Myeloperoxidase, a heme protein expressed in myeloid cells and possibly present in low levels in brain macrophages and microglial cells appears to be up-regulated in activated macrophages and microglial cells around demyelinating lesions in multiple sclerosis (15). Myeloperoxidase uses H2O2 to generate oxidizing intermediates that destroy pathogens. Recent studies indicate that this enzyme and other heme proteins can use H2O2 to oxidize nitrite, a decomposition product of NO, generating 3-NT and other nitration products in vitro (8, 16-18). Heme proteins also oxidize tyrosine to the tyrosyl radical, a reactive intermediate that promotes o,o-dityrosine formation. Incubation of brain proteins with the myeloperoxidase-nitrite-H2O2 system showed increases in 3-NT levels similar to those observed in MPTP-treated mice. These observations indicate that myeloperoxidase can promote o,o-dityrosine and 3-NT formation by two distinct pathways, one involving the tyrosyl radical and the other involving reactive nitrogen

species. These *in vitro* results and the protective effects afforded by NOS inhibition leads us to speculate that if myeloperoxidase does contribute to MPTP-induced toxicity, it does so through the pathway involving reactive nitrogen species. In the MPTP mouse model, a strong astrocytic and microglial response occurs in the striatum and ventral midbrain and soon after, DA neurons begin to die (19). This robust glial response may provide the necessary cellular substrate for myeloperoxidase induction followed by o,o-dityrosine production. According to this scenario, damage inflicted by the tyrosyl radical may be a secondary event in MPTP-induced toxicity. This does not undermine the pathological significance of myeloperoxidase action which could possibly peak during the most active phase of neurodegeneration (20). Therefore, the tyrosyl radical may not initiate but may enhance DA neuronal death in the MPTP mouse model of PD. Other heme proteins such as the mitochondrial heme protein cytochrome c can also catalyze selective increases in 3-NT and o,o-dityrosine in MPTP-treated mice which may be relevant to MPTP and PD because significant evidence points to the mitochondrion as the site of the major deleterious event that drives DA neurodegeneration.

The overall increase in oxidized amino acids in MPTP-induced brain injury represents about 2 in 10,000 protein tyrosine residues. This is a seemingly low level of oxidation products, therefore, is this really biologically relevant? The answers to this question lie in the fact that similar levels of oxidation products have been reported for other pathological conditions (6, 9, 21-27) and inherent in the method is substantial dilution of the targets of protein oxidation with proteins present in surrounding normal tissues. So, it is conceivable that the the overall level of protein oxidation will be low. Furthermore, there may be other targets for damage by reactive oxygen species such as lipids and DNA which may require measurement of other not yet known markers. And finally, our *in vitro* studies provide strong evidence that the pattern of oxidized amino acids serve as a molecular fingerprint for the pathways that are mediating oxidative damage in vivo (5, 28).

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Key Research Accomplishments for Specific Aim III.

- Demonstrated that 3-NT and o,o-dityrosine levels are significantly elevated in ventral midbrain and striatum of MPTP-treated C57/BL mice.
- Demonstrated that MPTP administration does not change ortho-tyrosine levels in the brain of MPTP-treated C57/BL mice.
- Measurement of 3-NT, o,o-dityrosine and ortho-tyrosine formation *in vitro* in brain tissues exposed to peroxynitrite.
- Demonstrated 3-NT, o,o-dityrosine and ortho-tyrosine formation *in vitro* in brain proteins oxidized in vitro by myeloperoxidase-generated tyrosyl radical.
- Demonstrated 3-NT, o,o-dityrosine and ortho-tyrosine formation *in vitro* in brain proteins oxidized by HO.-generating system containing copper and H2O2.
- Overall demonstration that 3-NT, o,o-dityrosine and ortho-tyrosine can be used as markers of protein oxidation and damage in the brain.

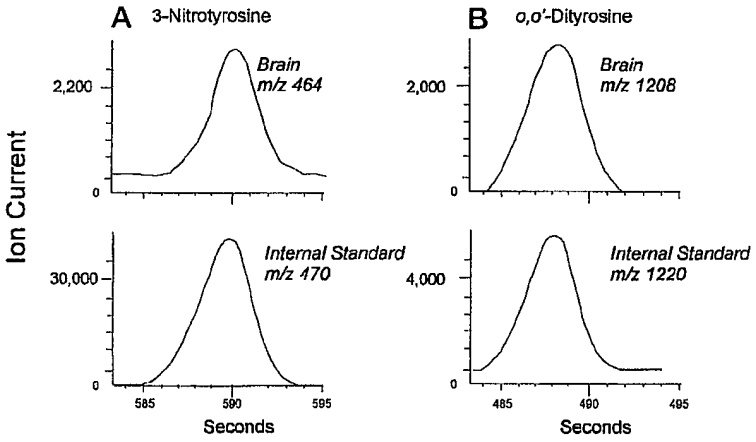

FIG. 1. **Detection of *n*-propyl heptafluorobutyryl derivatives of 3-nitrotyrosine (*A*) and *o,o'*-dityrosine (*B*) in mouse ventral midbrain by selected ion monitoring negative-ion electron capture GC/MS.** Tissue samples were delipidated, acid-hydrolyzed, and subjected to solid-phase extraction on a C-18 column. Isolated oxidized amino acids were derivatized and subjected to GC/MS analysis as described under "Experimental Procedures." Note co-elution of the major ion expected for (*A*) 3-nitrotyrosine (*m/z* 464) with that of 3-nitro[$^{13}C_6$]tyrosine (*m/z* 470) and (*B*) *o,o'*-dityrosine (*m/z* 1208) with that of *o,o'*-[$^{13}C_{12}$]dityrosine (*m/z* 1220).

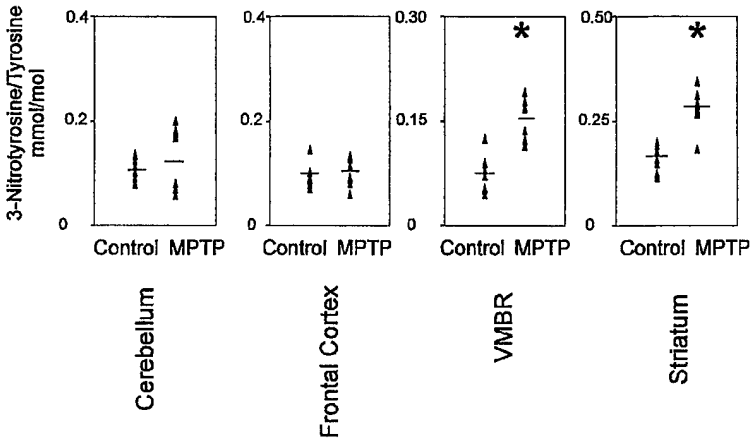

FIG. 2. **3-Nitrotyrosine in brain proteins isolated from control and MPTP-treated mice.** After addition of ^{13}C-labeled internal standards, tissue from the indicated region of the brain was delipidated, hydrolyzed, and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by negative-ion electron capture GC/MS with selected ion monitoring. Three independent analyses of tissue were performed on 8 control and 8 MPTP-treated animals for a total of 24 analyses for each area of the brain. Values are the mean ± S.E. and are normalized to tyrosine and phenylalanine, the precursor amino acids. *, $p < 0.05$ by analysis of variance.

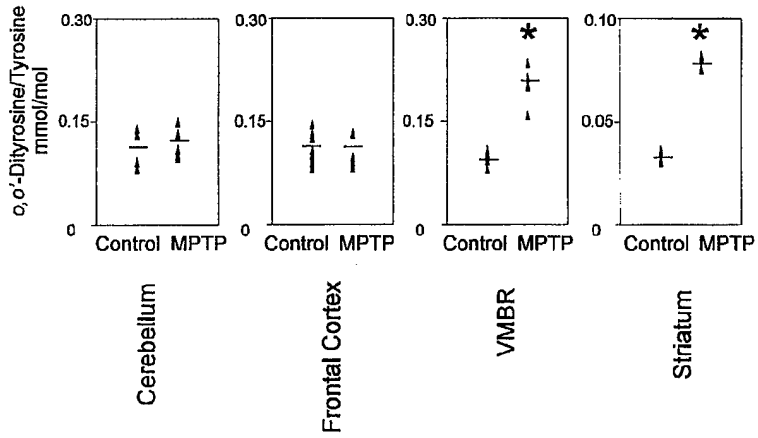

FIG. 3. ***o,o'*-Dityrosine in brain proteins isolated from control and MPTP-treated mice.** Tissue from the indicated region of brain in control and MPTP-treated mice was subjected to isotope dilution GC/MS analysis. Values are the mean ± S.E. of triplicate determinations from 8 control animals and 8 MPTP-treated animals. *, $p < 0.05$ by analysis of variance.

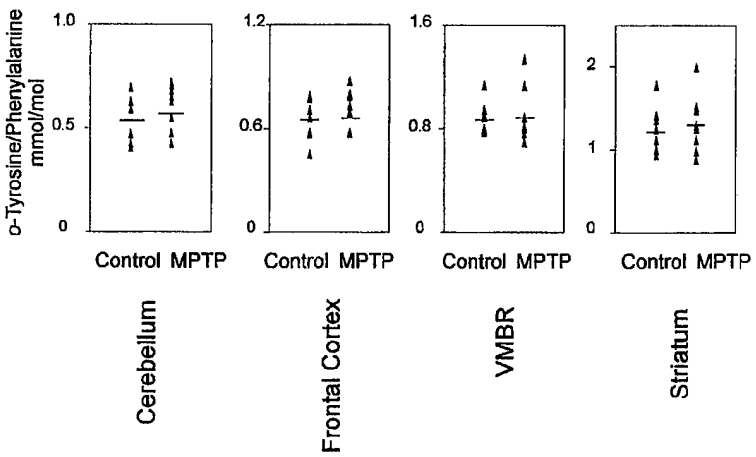

FIG. 4. ***ortho*-Tyrosine in brain proteins isolated from control and MPTP-treated mice.** Tissue from the indicated region of brain in control and MPTP-treated mice was subjected to isotope dilution GC/MS analysis. Values are the mean ± S.E. of triplicate determinations from 8 control animals and 8 MPTP-treated animals.

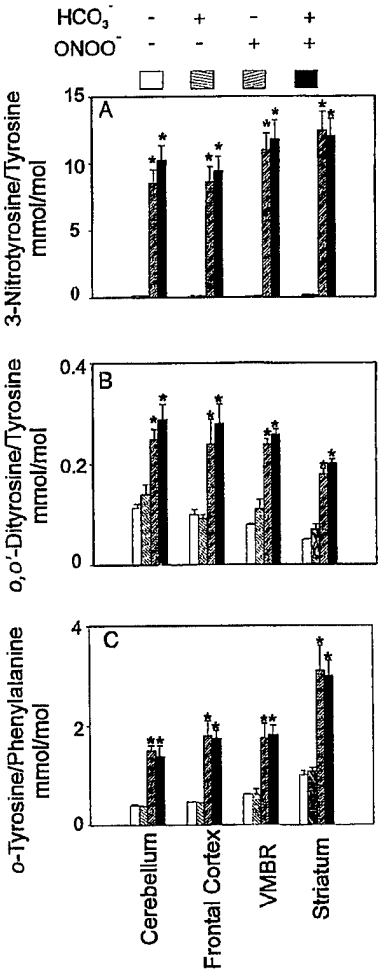

FIG. 5. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by peroxynitrite.** Proteins from the indicated areas of the brain were incubated for 10 min at 37 °C in buffer B alone (50 mM sodium phosphate, pH 7.4), buffer B with 25 mM HCO_3^-, buffer B supplemented with 1 mM $ONOO^-$, or buffer B with 1 mM $ONOO^-$ and 25 mM HCO_3^-. Reactions were initiated by the addition of $ONOO^-$. After acid precipitation and addition of ^{13}C-labeled internal standards, proteins were hydrolyzed and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by isotope dilution negative-ion electron capture GC/MS with selected ion monitoring. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$ by paired t test.

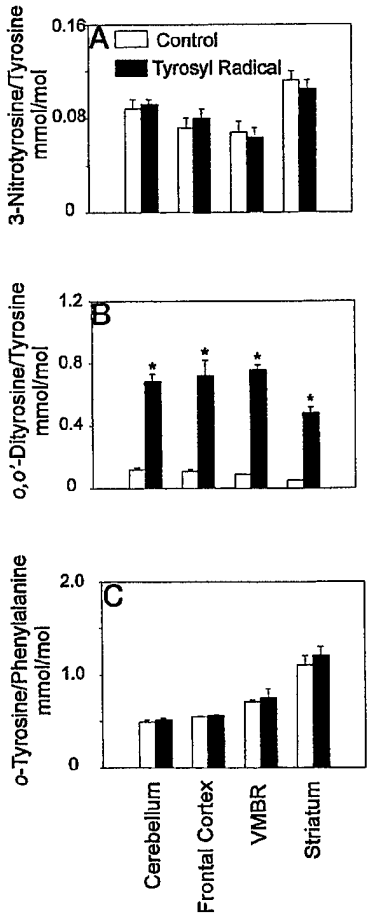

FIG. 6. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by myeloperoxidase-generated tyrosyl radical.** Proteins from the indicated areas of the brain were incubated for 30 min at 37 °C in buffer B containing 0.1 mM DTPA (*Control*) or buffer B supplemented with of 0.1 mM H_2O_2, 20 nM myeloperoxidase, 0.2 mM L-tyrosine, and 0.1 mM DTPA (*Tyrosyl Radical*). Levels of oxidized amino acids in tissue proteins were determined by isotope dilution GC/MS. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$.

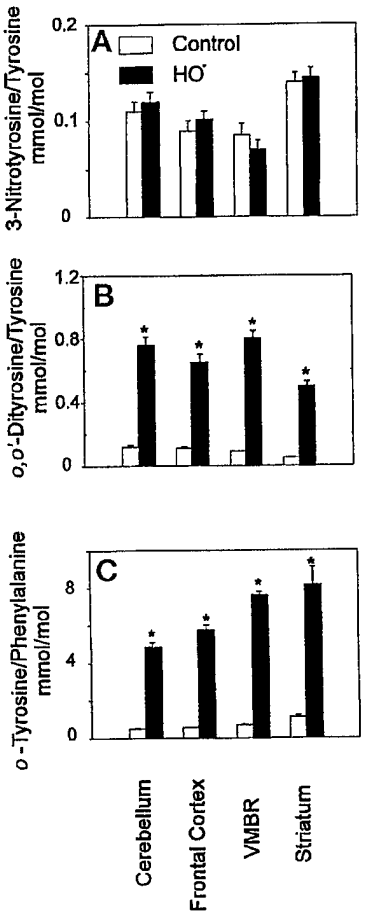

FIG. 7. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by hydroxyl radical.** Proteins from the indicated areas of the brain were incubated for 2 h at 37 °C in buffer B (*Control*) or buffer B supplemented with 0.2 mM $CuSO_4$ and 5 mM H_2O_2 ($HO^{\cdot}$). To inhibit residual tissue catalase activity, 0.1 mM 3-amino-1,2,4-triazole was included in reaction mixtures. Levels of oxidized amino acids in tissue proteins were determined by isotope dilution GC/MS. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$.

Specific Aim IV

Specific Aim IV was set forth to examine the potential biological consequences of protein tyrosine nitration by assessing whether the candidate proteins, mitochondrial electron transport chain polypeptides and manganese-superoxide dismutase are nitrated. This will be tested in PC-12 cells after exposure to different concentrations of and lengths of time to peroxynitrite and MPTP's active metabolite, MPP+ as well as in wild type and in transgenicSOD1 and knockout nNOS and iNOS mice after MPTP administration. Tyrosine nitration will be ascertained by immunoprecipitation, Western blot analysis and amino acid analysis. The catalytic activity of these enzymes in both PC-12 cells and mice experiments will also be undertaken by using photospectrometric enzymatic assays. We have not yet initiated measurement of the consequences of protein tyrosine nitration in PC-12 cells following exposure to MPP+ or in wild type and various lines of transgenic mice following MPTP administration as our PC-12 cell culture system has not been set up and the animals necessary for **Specific Aim IV** have to be bred to the needed numbers. We have begun to work out our methods for the purification of mitochondria and the mitochondrial fractions necessary for this part of the award.

Conclusions

MPTP presents a number of events that may actually mirror the events that occur in the neurodegenerative process in PD. This year's experiments provide compelling evidence that the source of NO production in the substantia nigra of the MPTP mouse model of PD is the up-regulation of the iNOS enzyme in activated microglia. We also demonstrate that an inflammatory-type response is induced by MPTP, an event which precedes the MPTP-induced DA neuronal death (previously described) as peak levels of the iNOS enzyme occur prior to the major cell death event seen in this MPTP mouse model of PD. iNOS up-regulation has been measured in post-mortem samples from PD brains and a strong glial response has been noted in these brains. Thus, our data support this NO finding in humans and further attests to the similarity between PD and the MPTP mouse model of PD. MPTP-induced events include the inactivation of tyrosine hydroxylase (TH), the rate-limiting enzyme in the production of DA. This year's experiments also demonstrate that proteins from brain areas known to be susceptible to the toxic effects of MPTP accumulate 3-nitrotyrosine, o,o-dityrosine and ortho-tyrosine, nitrated protein compounds that can inactivate TH and which can act as markers of MPTP-induced oxidative damage. Formation of these nitrated compounds may occur through the induction of the myeloperoxidase enzyme which may attack tyrosine residues in the TH enzyme in the presence of H2O2 to produce the tyrosyl radical or through reactive nitrogen species formed because of the increase in iNOS enzyme induction. It remains to be sorted out as to which pathway dominates. Nonetheless, the above data has probably inches us closer to a cause for PD.

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Mass Spectrometric Quantification of 3-Nitrotyrosine, *ortho*-Tyrosine, and *o,o'*-Dityrosine in Brain Tissue of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated Mice, a Model of Oxidative Stress in Parkinson's Disease*

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Oxidative stress is implicated in the death of dopaminergic neurons in Parkinson's disease and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease. Oxidative species that might mediate this damage include hydroxyl radical, tyrosyl radical, or reactive nitrogen species such as peroxynitrite. In mice, we showed that MPTP markedly increased levels of *o,o'*-dityrosine and 3-nitrotyrosine in the striatum and midbrain but not in brain regions resistant to MPTP. These two stable compounds indicate that tyrosyl radical and reactive nitrogen species have attacked tyrosine residues. In contrast, MPTP failed to alter levels of *ortho*-tyrosine in any brain region we studied. This marker accumulates when hydroxyl radical oxidizes protein-bound phenylalanine residues. We also showed that treating whole-brain proteins with hydroxyl radical markedly increased levels of *ortho*-tyrosine *in vitro*. Under identical conditions, tyrosyl radical, produced by the heme protein myeloperoxidase, selectively increased levels of *o,o'*-dityrosine, whereas peroxynitrite increased levels of 3-nitrotyrosine and, to a lesser extent, of *ortho*-tyrosine. These *in vivo* and *in vitro* findings implicate reactive nitrogen species and tyrosyl radical in MPTP neurotoxicity but argue against a deleterious role for hydroxyl radical in this model. They also show that reactive nitrogen species and tyrosyl radical (and consequently protein oxidation) represent an early and previously unidentified biochemical event in MPTP-induced brain injury. This finding may be significant for understanding the pathogenesis of Parkinson's disease and developing neuroprotective therapies.

Parkinson's disease (PD)[1] is attributed to a profound deficit in dopamine (1) that follows the loss of dopaminergic neurons in the substantia nigra pars compacta and dopaminergic nerve terminals in the striatum (2). Although the mechanisms are uncertain, a large body of experimental evidence implicates oxidative stress (reviewed in Refs. 3–5). In light of these human and animal studies, at least three pathways have been proposed.

Studies with the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD suggest a pivotal role for superoxide radical ($O_2^{\cdot-}$) both *in vitro* and *in vivo* (6–10). $O_2^{\cdot-}$ is not highly reactive, however, and is generally thought not to cause serious direct injury (4, 11). Instead, it is believed to exert many or most of its toxic effects by generating reactive species such as hydroxyl radical ($HO^{\cdot}$), whose oxidative properties can ultimately kill cells (4, 11). For instance, $O_2^{\cdot-}$ facilitates $HO^{\cdot}$ production in the metal-catalyzed Haber-Weiss reaction both by reducing redox-active transition metals (M^{n+}) and by dismutating to form hydrogen peroxide (H_2O_2) (4, 11).

$$M^{n+} + O_2^{\cdot-} \rightarrow M^{n+-1} + O_2$$
$$M^{n+-1} + H_2O_2 \rightarrow M^{n+} + HO^{\cdot} + HO^-$$

REACTIONS 1 AND 2

This noxious reaction may be relevant to PD because the postmortem concentration of nonheme iron is dramatically elevated in the substantia nigra pars compacta of PD patients (12, 13). Moreover, neuromelanin, believed to be a by-product of dopamine autooxidation, may promote the formation of reactive species such as $HO^{\cdot}$ through various mechanisms (14).

Superoxide can also react with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), another potent oxidant (15).

$$O_2^{\cdot-} + NO \rightarrow ONOO^-$$

REACTION 3

Under normal conditions, $O_2^{\cdot-}$ may be limiting, resulting in little $ONOO^-$ formation. Appreciable amounts could form if $O_2^{\cdot-}$ concentrations increased, as in the response to MPTP or PD, however. NO is implicated by the observation that nitric-oxide synthase inhibitors attenuate MPTP-induced dopaminergic

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[1] The abbreviations used are: PD, Parkinson's disease; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; GC/MS, gas chromatography-mass spectrometry; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; HPLC, high performance liquid chromatography.

neurotoxicity in mice and monkeys (16–18). Mutant mice deficient in neuronal nitric-oxide synthase also are resistant to MPTP (16).

While investigating oxidative damage in atherosclerosis and other inflammatory conditions (5, 19), we described an oxidative pathway that does not require free metal ions. It involves myeloperoxidase, a heme protein secreted by activated phagocytes (20). Myeloperoxidase uses H_2O_2 to convert the phenolic amino acid tyrosine into a reactive intermediate that promotes the oxidation of proteins and lipids (21–23). We have recently used electron paramagnetic resonance spectroscopy to demonstrate that the oxidizing intermediate generated by myeloperoxidase and other peroxidases is free tyrosyl radical (24). These studies support the idea that tyrosyl radical may promote oxidative reactions at sites of inflammation.

One powerful strategy for understanding the underlying mechanisms of oxidative injury is to identify stable end products of protein oxidation by different reaction pathways *in vitro* and then to determine whether these markers are present at elevated levels *in vivo* (19, 25). For instance, HO$^\cdot$ converts protein-bound phenylalanine residues to the unnatural amino acid isomer *ortho*-tyrosine. Tyrosyl radical forms *o,o'*-dityrosine as the major product, whereas $ONOO^-$ generates 3-nitrotyrosine. These compounds are stable to acid hydrolysis, making them potentially useful markers of protein oxidation *in vivo*.

Immunohistochemical and HPLC methods have been used to detect products such as 3-nitrotyrosine (26, 27), but both approaches may be confounded by structurally distinct molecules. Moreover, antibody-based analyses are semi-quantitative at best. HPLC can be more sensitive and specific than immunohistochemistry, but brain tissue may contain materials that interfere with this method (27). Therefore, the hypothesis that neuronally derived NO reacts with $O_2^{\cdot-}$ to generate $ONOO^-$ remains questionable, as does the idea that HO$^\cdot$ is produced in the MPTP model and PD.

Using isotope dilution gas chromatography-mass spectrometry (GC/MS), we developed sensitive and highly specific quantitative assays for measuring tissue levels of *ortho*-tyrosine, *o,o'*-dityrosine and 3-nitrotyrosine (25). We then analyzed the relative product yields of each marker in brain proteins oxidized *in vitro* by HO$^\cdot$, tyrosyl radical, or $ONOO^-$. We also assayed the oxidized amino acids in brain proteins from control and MPTP-treated mice. Our results suggest that reactive nitrogen species and tyrosyl radical, perhaps generated by $ONOO^-$ or heme proteins, mediate protein oxidation in this mouse model of PD.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from either Sigma or Aldrich unless otherwise specified. All organic solvents were HPLC grade. Cambridge Isotope Laboratories (Andover, MA) supplied ^{13}C-labeled amino acids for the preparation of internal standards. *o,o'*-[$^{13}C_{12}$]Dityrosine and *ortho*-[$^{13}C_6$]tyrosine were synthesized as described previously (21). 3-Nitro[$^{13}C_6$]tyrosine was synthesized using tetranitromethane (28). Concentrations of ^{13}C-labeled amino acids were determined by HPLC analysis (29).

Animals—Male C57/BL mice (8 weeks old; 22–25 g; Charles River Breeding Laboratories) were used in the study. Animals were housed three per cage in a temperature- and light-controlled room with a 12-h/12-h light-dark cycle. The mice were provided with water and food *ad libitum*. On the day of the experiment, mice received four intraperitoneal injections of MPTP-HCl (20 mg/kg; Research Biochemicals, Natick, MA) in saline every 2 h over an 8-h period. Control mice received saline only. All procedures followed National Institutes of Health guidelines for the use of live animals and were approved by the Columbia University Institutional Animal Care and Use Committee.

Collection of Tissues—Animals were anesthetized and sacrificed 24 h after the last injection; this time point was based on our previous study of nitration of tyrosine hydroxylase in the MPTP mouse model (30). To minimize *ex vivo* oxidation, mice were perfused with ice-cold antioxidant buffer (100 μM diethylenetriaminepentaacetic acid (DTPA; a metal chelator), 1 mM butylated hydroxytoluene (BHT; an inhibitor of lipid peroxidation), 10 mM 3-amino-1,2,4-triazole (an inhibitor of peroxidases and nitric-oxide synthase), 50 mM sodium phosphate, pH 7.4). Then, cerebellum, ventral midbrain, striatum, and cerebral cortex were dissected out on an ice-cold plate, frozen on dry ice, and stored at −80 °C until analysis (30).

Isolation of Brain Proteins for in Vitro Oxidation Studies—Freshly prepared sample from the indicated brain area was homogenized at 4 °C in 5 ml of buffer A (0.1 mM DTPA, pH 7.4), freeze-thawed once, and centrifuged at 10,000 × *g* for 10 min. The low speed supernatant was dialyzed against distilled, deionized water that had been passed over a Chelex resin (Bio-Rad) column to remove free metal ions.

Protein Oxidation by Hydroxyl Radical, Myeloperoxidase, and Peroxynitrite—*In vitro* oxidation reactions (1 mg brain protein/ml) were performed at 37 °C in buffer B (50 mM sodium phosphate, pH 7.4). When indicated, buffer B was supplemented with 25 mM $NaHCO_3$. Reactions were terminated by addition of 0.2 mM DTPA (pH 7.4), 300 nM catalase, and 0.1 mM BHT. Proteins were precipitated with ice-cold trichloroacetic acid (10% final concentration), acid-hydrolyzed, and subjected to GC/MS analysis. $ONOO^-$ was synthesized from 2-ethoxyethyl nitrite and H_2O_2 (31) and stored at −80 °C. $ONOO^-$ was thawed immediately prior to use, and its concentration was determined spectrophotometrically (ϵ_{302} = 1, 670 M^{-1} cm^{-1}; Ref. 32).

Amino Acid Isolation and Derivatization—All procedures were carried out at 4 °C. Brain tissue (~ 10 mg wet weight) was homogenized in 1 ml of antioxidant buffer. After dialysis overnight against buffer A, samples were delipidated by extraction with water/methanol/water-washed diethyl ether (1:3:7; v/v/v) for 10 min. Protein precipitate was recovered by centrifugation at 500 × *g* for 10 min. The resulting protein pellet was lipid extracted once more and dried under N_2. Samples (~1 mg of protein) were dried under vacuum in 1-ml glass reaction vials and immediately suspended in 0.5 ml of 6 N HCl (Sequenal grade, Pierce) containing 0.1% benzoic acid and 0.1% phenol (w/v). Isotopically labeled internal standards were added, and samples were hydrolyzed at 110 °C for 24 h under argon. Aromatic amino acids were isolated using a solid-phase C-18 column (3 ml; Supelclean SPE, Supelco Inc., Bellefonte, PA) (21) and converted to *n*-propyl esters by addition of 200 μl of HCl/*n*-propanol (prepared by the addition of one volume of 12 N HCl to three volumes of *n*-propanol) and heating for 1 h at 65 °C. After evaporation of excess reagent under N_2, heptafluorobutyric anhydride/ethyl acetate (1:3, v/v) was added, and the samples were heated at 65 °C for 15 min.

Mass Spectrometric Analysis—Amino acids were quantified using isotope dilution negative-ion electron capture GC/MS using a Hewlett Packard 5890 gas chromatograph interfaced with a Hewlett Packard 5988A mass spectrometer with extended mass range (21). Under these chromatography conditions, authentic compounds and isotopically labeled standards exhibited retention times identical to those of analytes derived from tissue samples. The limit of detection (signal/noise >10) was <1 pmol for all of the amino acids. The ions used for detecting analyte and internal standard were: phenylalanine, *m/z* 383 and 389 ions; tyrosine, *m/z* 417 and 423 ions; 3-nitrotyrosine, *m/z* 464 and 470 ions; *ortho*-tyrosine, *m/z* 595 and 601 ions; *o,o'*-dityrosine *m/z* 1208 and 1220 ions.

Statistical Analysis—Differences between two groups were compared using an unpaired Student's *t* test. Multiple comparisons were performed using a two-way analysis of variance. The null hypothesis was rejected at the 0.05 level.

RESULTS

Mass Spectrometric Detection of 3-Nitrotyrosine, o,o'-Dityrosine, and ortho-Tyrosine in Mouse Brain—To determine whether oxidized amino acids are present in normal mouse brain, we assayed freshly isolated tissue from frontal cortex, cerebellum, ventral midbrain and striatum. When amino acids from acid hydrolysates of each tissue were isolated and derivatized with *n*-propanol and heptafluorobutyric anhydride and then analyzed by GC/MS in the negative-ion electron capture mode, we detected compounds that exhibited major ions and retention times identical to those of authentic 3-nitrotyrosine, *ortho*-tyrosine, and *o,o'*-dityrosine. Selected ion monitoring demonstrated that the ions derived from the amino acids coeluted with ions derived from authentic ^{13}C-labeled internal

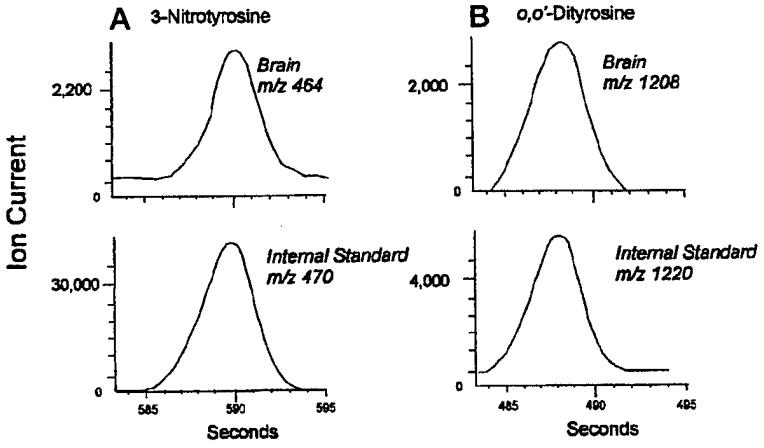

FIG. 1. **Detection of *n*-propyl heptafluorobutyryl derivatives of 3-nitrotyrosine (*A*) and *o,o'*-dityrosine (*B*) in mouse ventral midbrain by selected ion monitoring negative-ion electron capture GC/MS.** Tissue samples were delipidated, acid-hydrolyzed, and subjected to solid-phase extraction on a C-18 column. Isolated oxidized amino acids were derivatized and subjected to GC/MS analysis as described under "Experimental Procedures." Note co-elution of the major ion expected for (*A*) 3-nitrotyrosine (*m/z* 464) with that of 3-nitro[$^{13}C_6$]tyrosine (*m/z* 470) and (*B*) *o,o'*-dityrosine (*m/z* 1208) with that of *o,o'*-[$^{13}C_{12}$]dityrosine (*m/z* 1220).

standards, as shown in Fig. 1 for 3-nitrotyrosine and *o,o'*-dityrosine.

Levels of the oxidized amino acids varied both in absolute magnitude and in different parts of the brain (Fig. 2–4). All regions demonstrated relatively high levels of *ortho*-tyrosine (~ 0.5–1 mmol/mol of phenylalanine), which were highest in the striatum. In contrast, levels of 3-nitrotyrosine and *o,o'*-dityrosine were lower than those of *ortho*-tyrosine, and unlike *ortho*-tyrosine levels, those of *o,o'*-dityrosine were lowest in the striatum. However, 3-nitrotyrosine levels were comparable in all areas of the brain. These results indicate that acid hydrolysates of normal brain tissue proteins contain detectable levels of oxidized amino acids.

3-Nitrotyrosine Is Elevated in Ventral Midbrain and Striatum of MPTP-treated Mice—To determine whether MPTP promotes oxidative damage in brain proteins, we analyzed samples from eight control and eight MPTP-treated animals, comparing levels of oxidation products in the ventral midbrain and striatum, two regions that exhibit marked neuronal injury in MPTP-treated mice. We also analyzed samples from the frontal cortex and cerebellum, two regions of the brain that are little affected by MPTP. Tissue was prepared and analyzed by isotope dilution GC/MS as described above.

Levels of 3-nitrotyrosine in the ventral midbrain and striatum of the MPTP-treated animals were markedly higher (110% and 90%, respectively) than in the controls (Fig. 2). In contrast, there was no difference in level of 3-nitrotyrosine in the frontal cortex or cerebellum. These results indicate that levels of 3-nitrotyrosine increase selectively in the regions of the brain that are susceptible to the neurotoxic action of MPTP.

o,o'-Dityrosine Is Elevated in Ventral Midbrain and Striatum of MPTP-treated Mice—Levels of *o,o'*-dityrosine showed a strikingly similar pattern of increase as 3-nitrotyrosine (elevations of 120% and 170% compared with controls) in ventral midbrain and striatum, two regions of the brain that are vulnerable to MPTP-mediated neurotoxicity (Fig. 3). As with 3-nitrotyrosine, there was no difference in levels of *o,o'*-dityrosine in the frontal cortex or cerebellum. These results indicate that levels of *o,o'*-dityrosine increase selectively in the regions of the brain that are vulnerable to MPTP-mediated neuronal injury.

ortho-Tyrosine Levels Are Unchanged in All Regions of the Brain in MPTP-treated Mice—In contrast to 3-nitrotyrosine and *o,o'*-dityrosine, there was no change in the levels of *ortho*-tyrosine in any of the regions of the brain examined (Fig. 4). Collectively, these results indicate that levels of 3-nitrotyrosine and *o,o'*-dityrosine increase selectively in the regions of the brain that are susceptible to the neurotoxic action of MPTP. In contrast, *ortho*-tyrosine does not accumulate in increased amounts in any region of the brain examined in MPTP-treated animals.

3-Nitrotyrosine, o,o'-Dityrosine, and ortho-Tyrosine in Brain Tissue Oxidized by Peroxynitrite in Vitro—To evaluate the potential usefulness of 3-nitrotyrosine, *ortho*-tyrosine and *o,o'*-dityrosine as markers for oxidation *in vitro*, we investigated the product yield of these compounds in brain proteins that had been oxidized *in vitro* by $ONOO^-$, tyrosyl radical, and $HO^\cdot$. Protein used for the *in vitro* studies was isolated by centrifugation (10,000 × *g* for 10 min) from homogenate prepared from different regions of the brain. In brain proteins exposed to 1 mM $ONOO^-$, there was a dramatic increase (~ 80-fold) in 3-nitrotyrosine (Fig. 5*A*). Proteins isolated from different regions of the brain demonstrated similar increases after they were oxidized *in vitro* with $ONOO^-$. When brain protein was added 2 min after $ONOO^-$, however, protein nitration was minimal, indicating that $ONOO^-$ or a short-lived species derived from $ONOO^-$ nitrates the aromatic ring of protein-bound tyrosine (data not shown).

Because $ONOO^-$ also hydroxylates aromatic compounds and promotes cross-linking of phenolic groups (33), we determined whether *ortho*-tyrosine and *o,o'*-dityrosine form in brain proteins exposed to $ONOO^-$. Levels of *ortho*-tyrosine and *o,o'*-dityrosine increased 2–3-fold when we exposed brain protein to this reactive nitrogen species, but the product yields of *o,o'*-dityrosine and *ortho*-tyrosine were <5% and <25% that of 3-nitrotyrosine (Fig. 5, *B* and *C*). *In vitro* studies of model proteins exposed to a wide variety of different oxidation systems have previously demonstrated that 3-nitrotyrosine is a specific marker for protein oxidation by reactive nitrogen species (34).

Recent studies indicate that $ONOO^-$ rapidly reacts with carbon dioxide to generate $ONO_2CO_2^-$ (35–41). Bicarbonate is in equilibrium with carbonic acid that is present in extracellular fluids primarily as carbon dioxide, its conjugate acid. Bicarbonate/carbon dioxide is present at high concentrations *in vivo*, and the reactivity of $ONO_2CO_2^-$ differs from that of $ONOO^-$ (35–37). We therefore determined whether the addition of 25 mM $NaHCO_3$ to the reaction buffer affected the product yields of oxidized amino acids in brain proteins exposed to $ONOO^-$. The absolute increases and relative yields of 3-nitrotyrosine, *o,o'*-dityrosine, and *ortho*-tyrosine in brain proteins exposed to $ONOO^-$ were almost identical in the presence and absence of added bicarbonate/carbon dioxide (Fig. 5). The failure of $NaHCO_3$ to affect the product yields of the oxidized amino acids likely reflects the presence of bicarbonate in brain proteins and/or reaction mixture used for the experiments (35–41).

Taken together, these results indicate that 3-nitrotyrosine is

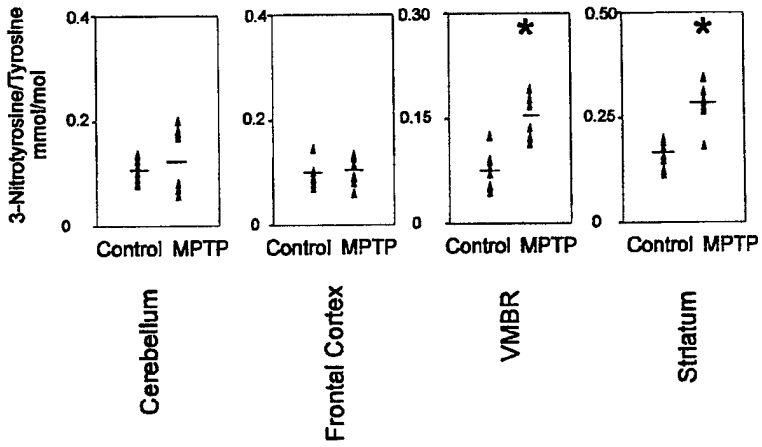

FIG. 2. **3-Nitrotyrosine in brain proteins isolated from control and MPTP-treated mice.** After addition of ^{13}C-labeled internal standards, tissue from the indicated region of the brain was delipidated, hydrolyzed, and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by negative-ion electron capture GC/MS with selected ion monitoring. Three independent analyses of tissue were performed on 8 control and 8 MPTP-treated animals for a total of 24 analyses for each area of the brain. Values are the mean ± S.E. and are normalized to tyrosine and phenylalanine, the precursor amino acids. *, $p < 0.05$ by analysis of variance.

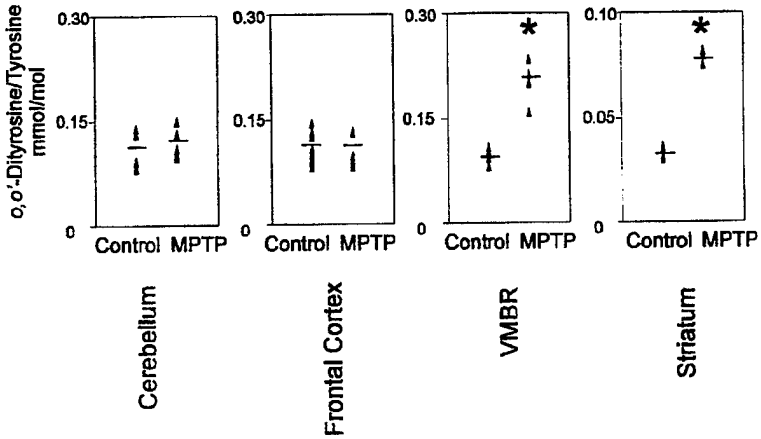

FIG. 3. ***o,o'*-Dityrosine in brain proteins isolated from control and MPTP-treated mice.** Tissue from the indicated region of brain in control and MPTP-treated mice was subjected to isotope dilution GC/MS analysis. Values are the mean ± S.E. of triplicate determinations from 8 control animals and 8 MPTP-treated animals. *, $p < 0.05$ by analysis of variance.

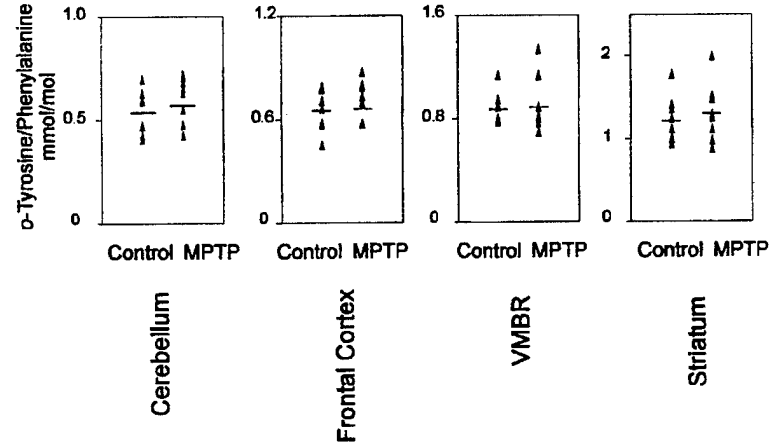

FIG. 4. ***ortho*-Tyrosine in brain proteins isolated from control and MPTP-treated mice.** Tissue from the indicated region of brain in control and MPTP-treated mice was subjected to isotope dilution GC/MS analysis. Values are the mean ± S.E. of triplicate determinations from 8 control animals and 8 MPTP-treated animals.

an excellent marker for proteins oxidized by reactive nitrogen species *in vitro*. They also suggest that *ortho*-tyrosine is a significant product of protein oxidation by $ONOO^-$. In contrast, *o,o'*-dityrosine is a relatively minor product.

3-Nitrotyrosine, o,o'-Dityrosine, and ortho-Tyrosine in Brain Protein Oxidized in Vitro by Myeloperoxidase-generated Tyrosyl Radical—We exposed protein derived from various areas of the brain to tyrosyl radical generated by the myeloperoxidase-tyrosine-H_2O_2 system, using physiologically plausible levels of oxidant and substrate (0.1 mM H_2O_2 and 0.2 mM tyrosine; Ref. 21). *o,o'*-Dityrosine was the major product of the reaction, with no change in levels of either 3-nitrotyrosine or *ortho*-tyrosine (Fig. 6). All regions of the brain exposed to tyrosyl radical showed a similar increase in *o,o'*-dityrosine. These results indicate that tyrosyl radical generated by myeloperoxidase selectively raises *o,o'*-dityrosine levels without changing levels of 3-nitrotyrosine or *ortho*-tyrosine.

Recent studies indicate that the myeloperoxidase-H_2O_2 system will convert tyrosine into 3-nitrotyrosine in a reaction that requires nitrite, (33) a degradation product of NO. Brain proteins incubated with myeloperoxidase, 0.1 mM H_2O_2, and 50 μM nitrite had 3-nitrotyrosine levels similar to those observed in MPTP-treated mice (Table I). These observations indicate that myeloperoxidase can promote the formation of *o,o'*-dityrosine and 3-nitrotyrosine by two distinct pathways, one involving tyrosyl radical and the other involving reactive nitrogen species.

3-Nitrotyrosine, o,o'-Dityrosine, and ortho-Tyrosine in Brain

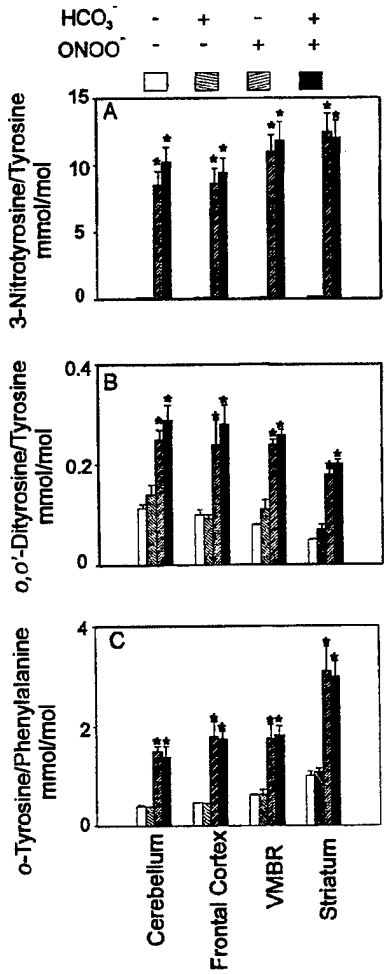

FIG. 5. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by peroxynitrite.** Proteins from the indicated areas of the brain were incubated for 10 min at 37 °C in buffer B alone (50 mM sodium phosphate, pH 7.4), buffer B with 25 mM HCO_3^-, buffer B supplemented with 1 mM $ONOO^-$, or buffer B with 1 mM $ONOO^-$ and 25 mM HCO_3^-. Reactions were initiated by the addition of $ONOO^-$. After acid precipitation and addition of ^{13}C-labeled internal standards, proteins were hydrolyzed and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by isotope dilution negative-ion electron capture GC/MS with selected ion monitoring. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$ by paired *t* test.

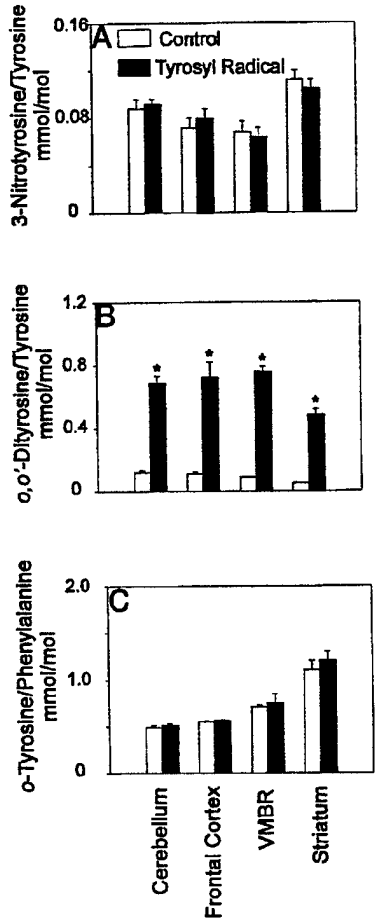

FIG. 6. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by myeloperoxidase-generated tyrosyl radical.** Proteins from the indicated areas of the brain were incubated for 30 min at 37 °C in buffer B containing 0.1 mM DTPA (*Control*) or buffer B supplemented with of 0.1 mM H_2O_2, 20 nM myeloperoxidase, 0.2 mM L-tyrosine, and 0.1 mM DTPA (*Tyrosyl Radical*). Levels of oxidized amino acids in tissue proteins were determined by isotope dilution GC/MS. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$.

Protein Oxidized by Hydroxyl Radical in Vitro—Proteins isolated from the cerebellum, frontal cortex, ventral midbrain, and striatum of control mice were incubated with a HO·-generating system (copper plus H_2O_2). We then determined levels of 3-nitrotyrosine, *o,o'*-dityrosine, and *ortho*-tyrosine in amino acid hydrolysates of the proteins using isotope dilution GC/MS. *ortho*-Tyrosine and *o,o'*-dityrosine accumulated in all four areas of the brain (Fig. 7, *A* and *B*). In contrast, the level of 3-nitrotyrosine did not change (Fig. 7*C*). The absolute increase in the level of *ortho*-tyrosine was ~10-fold higher than that of *o,o'*-dityrosine. It should be noted that copper plus H_2O_2 is a metal-catalyzed oxidation system that generates other reactive species in addition to HO· (42, 43). *In vitro* studies have demonstrated, however, that the relative product yields of *o,o'*-dityrosine and *ortho*-tyrosine are similar in model proteins exposed to copper plus H_2O_2 or ionizing radiation (a relatively pure source of HO·; Ref. 44). These observations suggest that *ortho*-tyrosine might serve as a marker for protein damage by HO· *in vivo*.

TABLE I
Product yield of 3-nitrotyrosine in brain proteins oxidized in vitro by the myeloperoxidase-H_2O_2-nitrite system

Proteins from the indicated area were incubated for 30 min at 37 °C in buffer B supplemented with 0.1 mM DTPA (Control) or buffer B supplemented with 20 nM myeloperoxidase (MPO), 0.1 mM H_2O_2, 50 μM nitrite, and 0.1 mM DTPA. Levels of protein-bound 3-nitrotyrosine were determined using isotope dilution GC/MS. Values are normalized to the content of the precursor amino acid tyrosine. Values are the mean ± S.E. of three determinations. Similar results were observed in two independent experiments. *, $p < 0.01$.

Brain area	3-Nitrotyrosine	
	Control	MPO-H_2O_2-nitrite
	μmol/mol	
Cerebellum	94 ± 16	313 ± 67*
Frontal cortex	79 ± 13	182 ± 16*
Ventral midbrain	84 ± 12	268 ± 60*
Striatum	195 ± 28	413 ± 42*

The concentrations of oxidant and redox catalysts were very different in the HO· and tyrosyl radical systems. There was 50

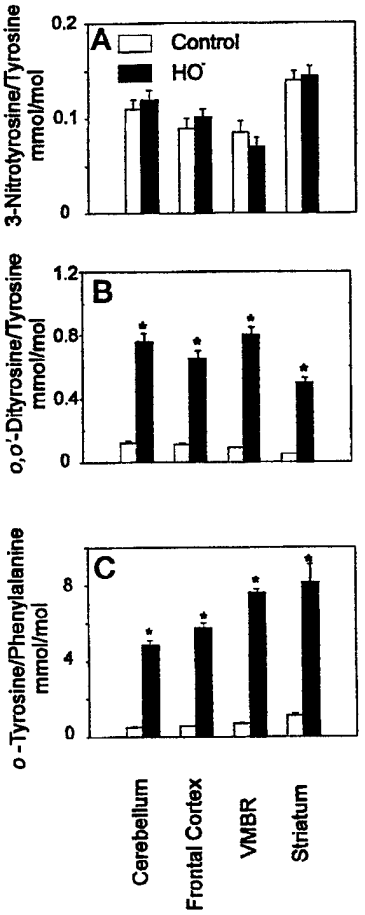

FIG. 7. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by hydroxyl radical.** Proteins from the indicated areas of the brain were incubated for 2 h at 37 °C in buffer B (*Control*) or buffer B supplemented with 0.2 mM $CuSO_4$ and 5 mM H_2O_2 ($HO^{\cdot}$). To inhibit residual tissue catalase activity, 0.1 mM 3-amino-1,2,4-triazole was included in reaction mixtures. Levels of oxidized amino acids in tissue proteins were determined by isotope dilution GC/MS. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$.

times as much H_2O_2 in the $HO^{\cdot}$ system as in the tyrosyl radical system (5 mM *versus* 0.1 mM), and 10,000 times as much copper as myeloperoxidase (200 μM *versus* 20 nM). In addition, proteins were exposed to the $HO^{\cdot}$ system for 4 times longer than the tyrosyl radical system. Despite these marked differences, the relative yields of *o,o'*-dityrosine were similar when brain protein was exposed to either $HO^{\cdot}$ or tyrosyl radical. These observations imply that, under our experimental conditions, myeloperoxidase-generated tyrosyl radical is much more efficient than $HO^{\cdot}$ at producing *o,o'*-dityrosine.

DISCUSSION

In the present studies, we used highly sensitive and specific isotope dilution mass spectrometric methods to investigate the pathways that oxidatively damage proteins in the brain under normal and pathological situations. We focused on three oxidants of potential pathophysiological significance: $ONOO^-$, a product of the interaction between $O_2^{\cdot-}$ and NO; tyrosyl radical generated by the heme protein myeloperoxidase; and $HO^{\cdot}$ radical generated by a metal-catalyzed oxidation system. We chose 3-nitrotyrosine, *ortho*-tyrosine, and *o,o'*-dityrosine as markers of protein oxidation because they are stable to acid hydrolysis and likely represent post-translational modifications of proteins.

In normal mice, we found detectable levels of 3-nitrotyrosine, *ortho*-tyrosine, and *o,o'*-dityrosine in all brain regions studied. This finding is consistent with the view that basal levels of oxidatively damaged proteins exist in physiological situations, especially in the brain. They probably result from the combination of the high rate of oxygen consumption and the poor oxidant-scavenging arsenal that characterize this organ. This "basal oxidative stress" seems to affect not only proteins, but DNA as well, since significant levels of 8-hydroxy-deoxyguanosine, a marker of oxidative damage to DNA, are present in normal rodent and human brains (45, 46). Collectively, these data support the idea that a mild but persistent oxidative stress may be normal and may lead, over time, to the decline in physiological functions that characterize aging (47). Interestingly, levels of the oxidation markers we assayed were not constant throughout the brain. In fact, we observed significant variations in 3-nitrotyrosine and *ortho*-tyrosine levels among the different brain regions studied, with highest levels in the striatum. The striatum also was among the brain regions showing the highest levels of 8-hydroxy-deoxyguanosine in both rats and humans (45, 46). These findings are in keeping with the belief that the striatum, and more broadly, the basal ganglia, are vulnerable to oxidative stress and frequently affected in oxidant-related neurodegenerative disorders (3).

Compared with saline-treated controls, the brains of MPTP-treated mice exhibited strikingly elevated levels of both *o,o'*-dityrosine and 3-nitrotyrosine. Moreover, we observed significant increases in these two markers in striatum and in ventral midbrain but not in frontal cortex and cerebellum, which is consistent with the specificity of MPTP's neurotoxic effects on the nigrostriatal dopaminergic pathway. These observations indicate that MPTP promotes protein oxidation specifically in brain regions known to be affected by the toxin and provide evidence that it stimulates both the production of reactive species and oxidative damage to critical cellular elements.

ortho-Tyrosine was the major product when brain proteins were exposed *in vitro* to $HO^{\cdot}$ generated by a metal-catalyzed oxidation system. In contrast, *ortho*-tyrosine concentrations were not altered in brain tissue from MPTP-treated mice. MPTP has been proposed to enhance $HO^{\cdot}$ production by impairing mitochondrial respiration (17, 48, 49). However, the lack of a detectable increase in *ortho*-tyrosine levels in the brain of MPTP-treated mice argues against a prominent role for $HO^{\cdot}$ in protein damage and casts doubt on the participation of $HO^{\cdot}$ in MPTP-induced neurotoxicity *in vivo*.

The marked increase in 3-nitrotyrosine levels after MPTP administration strongly suggests that reactive nitrogen intermediates play key roles in protein oxidation. Compelling evidence for this view comes from our *in vitro* experiments, which show that $ONOO^-$ causes a striking elevation of 3-nitrotyrosine, with a significant increase in *ortho*-tyrosine and a much smaller increase in *o,o'*-dityrosine. Our observations are in good agreement with previous demonstrations that inhibition of nitric-oxide synthase attenuates MPTP-induced dopaminergic toxicity (16–18).

Beal and collaborators showed that MPTP significantly increases striatal levels of free 3-nitrotyrosine in mice (17). However, the relationship between free 3-nitrotyrosine and 3-nitrotyrosine in proteins is unknown, and the pathophysiological significance, if any, of free 3-nitrotyrosine remains to be determined. In contrast, protein nitration may be deleterious because it alters the pK_a of tyrosine and can affect the protein's secondary and tertiary organization, thereby altering its function, as we have demonstrated for tyrosine hydroxylase (30).

Therefore, our data not only confirm that MPTP increases brain levels of 3-nitrotyrosine, but, more importantly, they strongly support the hypothesis that tyrosine nitration of brain proteins may play a critical role in the MPTP neurotoxic process.

The *o,o'*-dityrosine increase we observed after MPTP administration suggests that a pathway involving tyrosyl radical may provide a second physiologically relevant mechanism for protein oxidation in this model. In all cases where the biochemical pathway is known, a heme protein mediates *o,o'*-dityrosine synthesis. One such candidate is myeloperoxidase, which is specifically expressed by myeloid cells and may be present at low levels in brain macrophages and microglial cells. It also appears to be up-regulated in activated macrophage/microglial cells around demyelinating lesions in multiple sclerosis (50).

Myeloperoxidase uses H_2O_2 to generate oxidizing intermediates that destroy invading pathogens. Recent studies indicate that this enzyme and other heme proteins can use H_2O_2 to oxidize nitrite, a decomposition product of NO, generating 3-nitrotyrosine and other nitration products *in vitro* (33, 51–53). Heme proteins also oxidize tyrosine to tyrosyl radical, a reactive intermediate that promotes *o,o'*-dityrosine formation. Brain proteins incubated with the myeloperoxidase-nitrite-H_2O_2 system demonstrated an increase in 3-nitrotyrosine levels similar to those observed in MPTP-treated mice. These observations indicate that myeloperoxidase can promote the formation of *o,o'*-dityrosine and 3-nitrotyrosine by two distinct pathways, one involving tyrosyl radical and the other involving reactive nitrogen species. In light of these *in vitro* oxidation patterns and the protective effects of nitric-oxide synthase inhibitors, we speculate that if myeloperoxidase or other heme proteins contribute to the neurotoxicity of MPTP, they act through the pathway involving reactive nitrogen species.

In the MPTP model, a strong astrocytic and microglial reaction occurs in both the striatum and ventral midbrain soon after dopamine neurons begin to die (54). This robust glial reaction might provide the necessary cellular substrate for myeloperoxidase induction and therefore for *o,o'*-dityrosine production. According to this scenario, damage inflicted by tyrosyl radical may be a secondary event in MPTP-induced neurotoxicity. This does not, however, undermine the pathological significance of myeloperoxidase action, which would peak during the most active phase of neurodegeneration (55). Therefore, tyrosyl radical may not initiate but may enhance dopaminergic neuronal death in the MPTP mouse model of PD. Other heme proteins that can catalyze the selective increase in 3-nitrotyrosine and *o,o'*-dityrosine in the brains of MPTP-treated mice include mitochondrial heme proteins like cytochrome *c*. The latter might be especially relevant to both MPTP and PD because a large body of evidence points to the mitochondrion as the site of the major deleterious event that drives dopaminergic neurodegeneration.

The overall increase in oxidized amino acids in MPTP-induced brain injury represents ~2 in 10,000 protein tyrosine residues. One could raise the question of whether this low level of oxidation products is likely to be biologically important. We believe that the increases in specific oxidation products we observe may be significant for several reasons. First, similar levels of oxidation products have been reported in other conditions where oxidative damage has been implicated in the pathogenesis of disease (21, 34, 56–62). Second, an inherent problem with the analysis of biological material is that there is substantial "dilution" of the targets of protein oxidation with proteins present in surrounding normal tissue. Therefore, even with substantial damage to specific proteins it is conceivable that the overall level of protein oxidation products will be low. Indeed, the selective nitration of tyrosine hydroxylase by reactive nitrogen species has been proposed to be of critical pathologic importance in the pathogenesis of PD (30). Third, there could be other targets for damage by reactive oxidant species such as lipids and DNA that are biologically relevant. In this sense, the products we have quantified may be serving as markers of oxidative damage. Finally, we believe that our *in vitro* studies provide strong evidence that the pattern of elevation of oxidized amino acids serves as a "molecular" fingerprint for the pathways that are mediating oxidative damage *in vivo* (19, 63). Though the oxidation products themselves may or may not be pathophysiologically significant, the increases in 3-nitrotyrosine and *o,o'*-dityrosine we observe point toward reactive nitrogen species and tyrosyl radical as being physiologically relevant. In contrast, there is no evidence that HO$^{\cdot}$ (the oxidant generally proposed to be mediating protein damage) is playing a role in this model of PD.

Because of the similarity between the MPTP model and PD, it is possible that the culprits identified above may underlie the oxidative attack that presumably kills dopaminergic neurons in PD. Accordingly, the present study may have important implications for understanding the pathogenesis of PD and for developing antioxidant interventions to halt or retard neurodegeneration in this disorder.

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Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease

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MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) damages dopaminergic neurons as seen in Parkinson disease. Here we show that after administration of MPTP to mice, there was a robust gliosis in the substantia nigra pars compacta associated with significant upregulation of inducible nitric oxide synthase (iNOS). These changes preceded or paralleled MPTP-induced dopaminergic neurodegeneration. We also show that mutant mice lacking the iNOS gene were significantly more resistant to MPTP than their wild-type littermates. This study demonstrates that iNOS is important in the MPTP neurotoxic process and indicates that inhibitors of iNOS may provide protective benefit in the treatment of Parkinson disease.

Parkinson disease (PD) is a common neurodegenerative disorder whose cardinal features include tremor, slowness of movement, stiffness and poor balance[1]. Most, if not all, of these disabling symptoms are due to a profound reduction in striatal dopamine content caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and of their projecting nerve fibers in the striatum[2,3]. Although several approved drugs do alleviate PD symptoms, their chronic use is often associated with debilitating side effects[4], and none diminish the progression of the disease. Moreover, the development of effective neuroprotective therapies is impeded by our limited knowledge of the actual mechanisms by which dopaminergic neurons die in PD. So far, however, considerable insights into the pathogenesis of PD have been achieved by the use of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which causes in humans and in nonhuman primates a severe and irreversible PD-like syndrome[5]. In several mammalian species, MPTP reproduces most of the biochemical and pathological hallmarks of PD, including the substantial degeneration of dopaminergic neurons[5]. Furthermore, there is mounting evidence that reactive oxygen species, especially nitric oxide (NO), are pivotal in the MPTP neurotoxic process[6], which supports the hypothesis that oxidative stress contributes to the pathogenesis of PD (ref. 7).

So far, three distinct NO-synthesizing isoenzymes have been purified and molecularly cloned[8]: neuronal NO synthase (nNOS), inducible NOS (iNOS) and endothelial NOS. nNOS is the main NOS isoform in the brain, as its catalytic activity and protein are identifiable throughout the central nervous system[9,10]. In contrast, iNOS normally is not[11] or is minimally[12] expressed in the brain. However, in pathological conditions, iNOS expression can increase in brain glial cells[13] and invading macrophages in response to a variety of injuries[13–15]. Endothelial NOS is mainly localized in the endothelium of blood vessels and to a minimal extent in different discrete regions of the brain[16–18]. Thus, our success in determining the pathogenesis of PD as well as in developing neuroprotective therapies that target the NO pathway is contingent on our elucidation of which of the NOS isoenzymes contribute to the production of the NO involved in dopaminergic neuron degeneration. The pharmacological inhibition of nNOS, produced by 7-nitroindazole and *S*-methylthiocitrulline, substantially attenuates MPTP-induced dopaminergic neurotoxicity in mice and monkeys[19–22]. Although these NOS antagonists are considered selective nNOS inhibitors, it is not certain that, at the dose used in these studies, they retain all of their selectivity. Consistent with this is the demonstration that nNOS-deficient mice with about 10% residual nNOS activity[10] are partially protected against MPTP (ref. 20), whereas mice treated with doses of 7-nitroindazole that cause about 80% nNOS inhibition are completely protected[19,20]. These data indicate that although nNOS is important, other NOS isoforms might also participate in the dopaminergic neurodegeneration that occurs in the MPTP model and in PD. Relevant to this is the demonstration that many cells in the SNpc from postmortem PD samples express considerable amounts of iNOS, whereas those from age-matched controls do not[23]. Although upregulation of iNOS in acute injury may lead to cell death[15,24], most likely through the production of large amounts of NO over a prolonged period of time[25], its involvement in a chronic neurodegenerative process such as in PD is not known. Here we show that iNOS is not only upregulated in the SNpc of MPTP-treated mice, but that its ablation in mutant mice significantly attenuates MPTP neurotoxicity, thus indicating that iNOS is essential in MPTP-induced SNpc dopaminergic neurodegeneration.

Table 1 Number of neurons in the SNpc

	Saline		MPTP	
	Wild-type	*iNOS*$^{-/-}$	Wild-type	*iNOS*$^{-/-}$
Tyrosine hydroxylase	10,188 ± 619	9,680 ± 496	2,940 ± 698*	5,720 ± 539**
Nissl	13,563 ± 1007	12,913 ± 530	6,300 ± 539*	10,417 ± 380**

SNpc neurons (mean ± s.e.m.; $n = 5$ per group) were counted by stereology. *, $P < 0.001$, fewer than both saline-injected groups; **, $P < 0.05$, fewer than both saline-injected groups and more than MPTP-injected wild-type mice; Newman-Keuls post-hoc test.

MPTP produces a robust glial response

In saline-injected mice, ventral midbrain expression of macrophage antigen-1 (MAC-1) and glial fibrillary acidic protein (GFAP), which are specific markers of microglia and astrocytes, respectively, was minimal (Fig. 1*a–c*). This corresponded to only a few faintly immunoreactive resting microglia and astrocytes in the substantia nigra (Fig. 1*f* and *i*). In MPTP-injected mice, ventral midbrain expression of MAC-1 and GFAP was significantly greater (Fig. 1*a–c*) and there were many robustly immunoreactive MAC-1-positive activated microglia and GFAP-positive reactive astrocytes (Fig. 1*d,e,g and h*). Although the MPTP-induced glial response predominated in the SNpc, it spanned the entire substantia nigra (Fig. 1*d* and *g*). Changes in MAC-1 expression were evident by 12 hours, reached a maximum by 24–48 hours, and were no longer different from control samples by day 7 after MPTP injection (Fig. 1*a* and *b*). In contrast to MAC-1 expression, changes in GFAP expression were only noticeable by 24 hours, were maximal by 4–7 days, and showed a trend towards returning to control levels by 21 days after MPTP injection (Fig. 1*a* and *c*). In the striatum, the time course of the MPTP-induced changes in MAC-1 and GFAP expression were similar to those seen in the ventral midbrain (data not shown).

MPTP stimulates iNOS expression in glial cells

In saline-injected mice, there were rarely iNOS-immunoreactive cells in the SNpc (Fig. 2). In MPTP-injected mice, the number of SNpc iNOS-positive cells increased rapidly over time, reaching a 250% increase by 24 hours after MPTP injection (Fig. 2). However, iNOS-positive cell counts were no longer significantly different from controls by 48 hours after MPTP injection (Fig. 2*d*). In the striatum, no iNOS-positive cells were identified after MPTP injection. We confirmed the specificity of the antibody against iNOS by western blot analysis (Fig. 2*e*).

To elucidate the nature of the SNpc iNOS-positive cells, we simultaneously immunostained midbrain sections for iNOS and MAC-1 or GFAP. At 24 hours after MPTP injection, the time with the most iNOS-positive cells, there was iNOS immunoreactivity in MAC-1-positive activated microglial cells (Fig. 2*c*). In contrast, none of the iNOS-positive cells were either GFAP-positive or had a neuronal morphology.

MPTP increases iNOS mRNA levels and enzymatic activity

We further characterized the iNOS response to MPTP by assessing its mRNA level and enzymatic activity. In saline-injected mice, ventral midbrain iNOS mRNA was almost undetectable (Fig. 2). In contrast, in MPTP-injected mice, midbrain iNOS mRNA levels were detected by 12 hours, were maximal by 48 hours, and were no longer detected by 4 days after MPTP injection (Fig. 2*f* and *g*). Striatal iNOS mRNA levels were very low and were unchanged by MPTP injection (not shown). In agreement with the mRNA results, ventral midbrain iNOS enzymatic activity was minimal in saline-injected mice, but rapidly increased after MPTP injection (Fig. 2*h*). Indeed, in MPTP-injected mice, ventral midbrain iNOS activity began to increase by 12 hours, peaked by 24–48 hours (300% increase), and then slowly subsided back to control activity by 7 days after MPTP injection (Fig. 2*h*). As for iNOS mRNA, iNOS catalytic activity in the striatum was low and was unaffected by MPTP injection (not shown). We also measured nNOS activity in ventral midbrain from mice treated with saline or MPTP; nNOS activity was consistently higher than iNOS and was unmodified by MPTP injection (Fig. 2*h*).

iNOS-deficient mice are more resistant to MPTP

Given the MPTP-induced SNpc iNOS upregulation, we determined the involvement of this enzyme in MPTP neurotoxicity by comparing the effects of the toxin in mutant mice deficient in iNOS (*iNOS*$^{-/-}$) and in their wild-type littermates; this approach is more advantageous than pharmacological inhibition of NOS because it allows for the study of iNOS independently of other NOS isoenzymes. Stereological counts of SNpc dopaminergic neurons, defined by tyrosine hydroxylase (TH) and Nissl staining, did not differ between saline-injected *iNOS*$^{-/-}$ mice and their saline-injected wild-type littermates (Fig. 3*a* and Table 1). In wild-type mice, only 29% of the SNpc TH-positive neurons and 46% of the Nissl-stained SNpc neurons survived MPTP injection (Fig. 3*a* and Table 1). In contrast, about twice as many SNpc TH-positive and Nissl-stained neurons survived in *iNOS*$^{-/-}$ mice treated with an identical MPTP regimen (Fig. 3*a* and Table 1). However, there were no significant differences in the extent of loss in striatal levels of dopamine, DOPAC (3-4-dihydroxyphenylacetic acid) and HVA (homovanillic acid) between *iNOS*$^{-/-}$ mice and their wild-type littermates after the administration of MPTP (Table 2).

Microglial responses and MPP$^+$ production in *iNOS*$^{-/-}$

Although iNOS$^{-/-}$ mice lack iNOS expression, they showed increases in MAC-1 expression similar to those seen in wild-type mice in response to MPTP (Fig. 3*b* and *c*). The main determining factor of MPTP neurotoxic potency is its conversion in glia to the 1-methyl-4-phenylpyridinium ion^{26} (MPP$^+$). To confirm that the resistance of *iNOS*$^{-/-}$ mice was due to the absence of the iNOS gene and not due to an alteration in the glial production of MPP$^+$, we measured its striatal content at different times after MPTP injection. At no time did the striatal content of MPP$^+$ differ significantly between the *iNOS*$^{-/-}$ mice and their wild-type littermates (Table 3).

Table 2 Striatal monoamine levels (ng/mg tissue)

	Dopamine	DOPAC	HVA
Saline ($n = 6$)	14.7 ± 0.8	1.6 ± 0.3	2.9 ± 0.1
MPTP			
Wild-type ($n = 4$)	2.8 ± 0.5*	0.7 ± 0.2*	1.8 ± 0.2*
iNOS$^{-/-}$ ($n = 5$)	2.4 ± 0.3**	0.5 ± 0.1**	1.7 ± 0.2**

As dopamine, DOPAC, and HVA values did not differ between saline-injected *iNOS*$^{-/-}$ and their saline-injected wild-type littermates, data from both groups were combined. *, $P < 0.01$, different from saline-injected control mice but not MPTP-injected *iNOS*$^{-/-}$ mice; **, $P < 0.01$, different from saline-injected control mice but not MPTP-injected wild-type mice; Newman-Keuls post-hoc test. Data represent means ± s.e.m. for four to six mice per group.

Brain nitrotyrosine levels in *iNOS*$^{-/-}$ and wild-type mice

To assess the extent of NO-related oxidative damage, we determined nitrotyrosine levels by dot–blot analysis in selected brain regions of *iNOS*$^{-/-}$ and wild-type mice after saline or MPTP injection. In saline-injected mice, the distribution of nitrotyrosine was similar between the two groups of mice in that levels were highest in striatum and cerebellum, followed by frontal cortex, and were lowest in ventral midbrain (Table 4). In MPTP-injected *iNOS*$^{-/-}$ and wild-type mice, nitrotyrosine levels were significantly increased in striatum and ventral midbrain and unchanged in the other brain regions studied (Table 4). MPTP produced significantly smaller increases in nitrotyrosine levels in the ventral midbrain of *iNOS*$^{-/-}$ mice than of their wild-type counterparts, whereas it produced similar increases in the striata of the two groups of mice (Table 4).

Table 3 MPP$^+$ levels (μg/g striatum)

	90 min	120 min	180 min	360 min
Wild type	17.75 ± 0.50	23.10 ± 4.30	19.17 ± 0.63	1.61 ± 0.12
iNOS$^{-/-}$	24.24 ± 2.70	20.61 ± 2.23	17.18 ± 0.83	1.91 ± 0.49

Striatal MPP$^+$ levels in wild-type and *iNOS*$^{-/-}$ mice at 90, 120, 180 and 360 min after the last MPTP injection do not differ ($P > 0.05$; Newman-Keuls post-hoc test) between the two groups. Data represent means ± s.e.m. for four mice per group and time point.

Discussion

This study shows that, in addition to the considerable loss of dopaminergic neurons, gliosis is a salient neuropathological feature of the SNpc and the striatum in the MPTP mouse model, as in PD (refs. 27,28). Although gliosis sometimes may be associated with beneficial effects, there are many more situations in which gliosis may be deleterious[29,30], including in PD (ref. 28). Consistent with this, our data indicate that inflammatory-related events, such as gliosis, may contribute to the degeneration of dopaminergic neurons in the MPTP model. For example, activated microglial cells appeared in the SNpc much sooner than reactive astrocytes (Fig. 1) and at a time when only minimal neuronal death has occurred[31]. This supports the contention that the microglial response to MPTP arises early enough in the neurodegenerative process to contribute to the demise of SNpc dopaminergic neurons. In keeping with the deleterious role of microglia, we found that these cells not only increase in number after MPTP injection, but also, more importantly, were the site of iNOS upregulation (Fig. 2). Therefore, activated microglial cells can flood surrounding dopaminergic neurons with large amounts of iNOS-derived NO and other reactive species, such as superoxide radicals[32]. The time course of the response of astrocytes to MPTP was quite distinct from that of microglia (Fig. 1), in that the changes in the density of reactive astrocytes in both striatum and SNpc did not precede but rather occurred at the same time as the active phase of dopaminergic neuron degeneration[31]. This indicates that the astrocytic reaction is secondary to the loss of dopaminergic neurons and not a primary event. Although this diminishes the potential role of reactive astrocytes in initiating the dopaminergic neurodegeneration, it does not undermine the potential role of these cells in propagating the neurodegenerative process.

Both *in vitro* and *in vivo* experiments indicate that iNOS transcription can be induced by various cytokines, including tumor necrosis factor-α, interleukin-1β and interferon-γ (refs. 33–35) as well as by ligation of the macrophage cell surface antigen CD-23 (ref. 36). It is thus particularly relevant to PD that glial cells immunoreactive for those cytokines and CD-23 are detected in the SNpc of PD patients[37]. In agreement with this transcriptional induction model of iNOS, the mRNA levels of iNOS increased in the ventral midbrain of MPTP-injected mice (Fig. 2). However, in the striatum, whereas a strong glial reaction did occur after MPTP injection, there was no detectable induction of iNOS mRNA. Among various possibilities, the discrepancy in the iNOS response between striatum and ventral midbrain may reflect either a differential mode of iNOS regulation between these two brain regions or the existence of a striatal factor that suppresses the induction of iNOS[38].

Our data on microglial iNOS immunoreactivity in the SNpc of MPTP-injected mice are in agreement with results in PD patients, in whom iNOS immunoreactivity has also been found

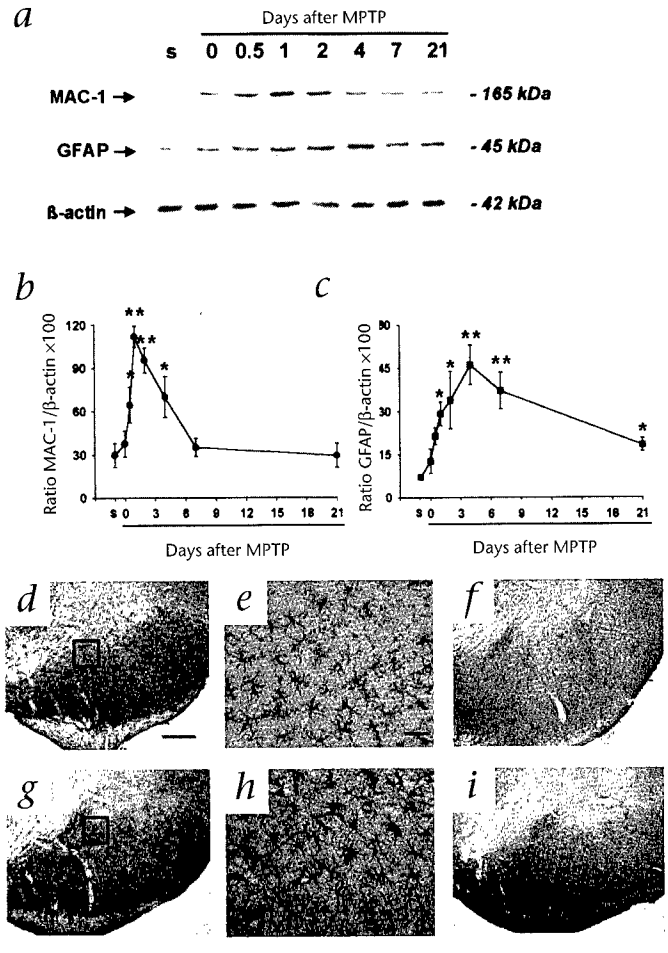

Fig. 1 MPTP-induced glial reaction. ***a–c***, Ventral midbrain MAC-1 (*a* and *b*) and GFAP (*a* and *c*) expression is minimal in saline-injected mice (S), but increases in a time-dependent manner after MPTP injection. Data represent mean ± s.e.m. ($n = 4–5$). **, $P < 0.01$ and *, $P < 0.05$, compared with saline, Newmas-Keuls post-hoc test. ***d–i***, There is a robust MAC-1 (*d*) and GFAP (*g*) immunostaining in the SNpc of MPTP-treated mice compared with that in saline-treated control mice (*f* and *i*) at 24 h after injection. *e* and *h*, Magnification of the boxed areas in *d* and *g* shows that the MAC-1- and GFAP-immunoreactive cells in the MPTP-treated mice seem to have a morphology typical of activated microglia cells (*e*) and of reactive astrocytes (*h*). Scale bars represent 200 μm (*d,f,g,i*; shown in *d*) and 15 μm (*e,h*; shown in *e*).

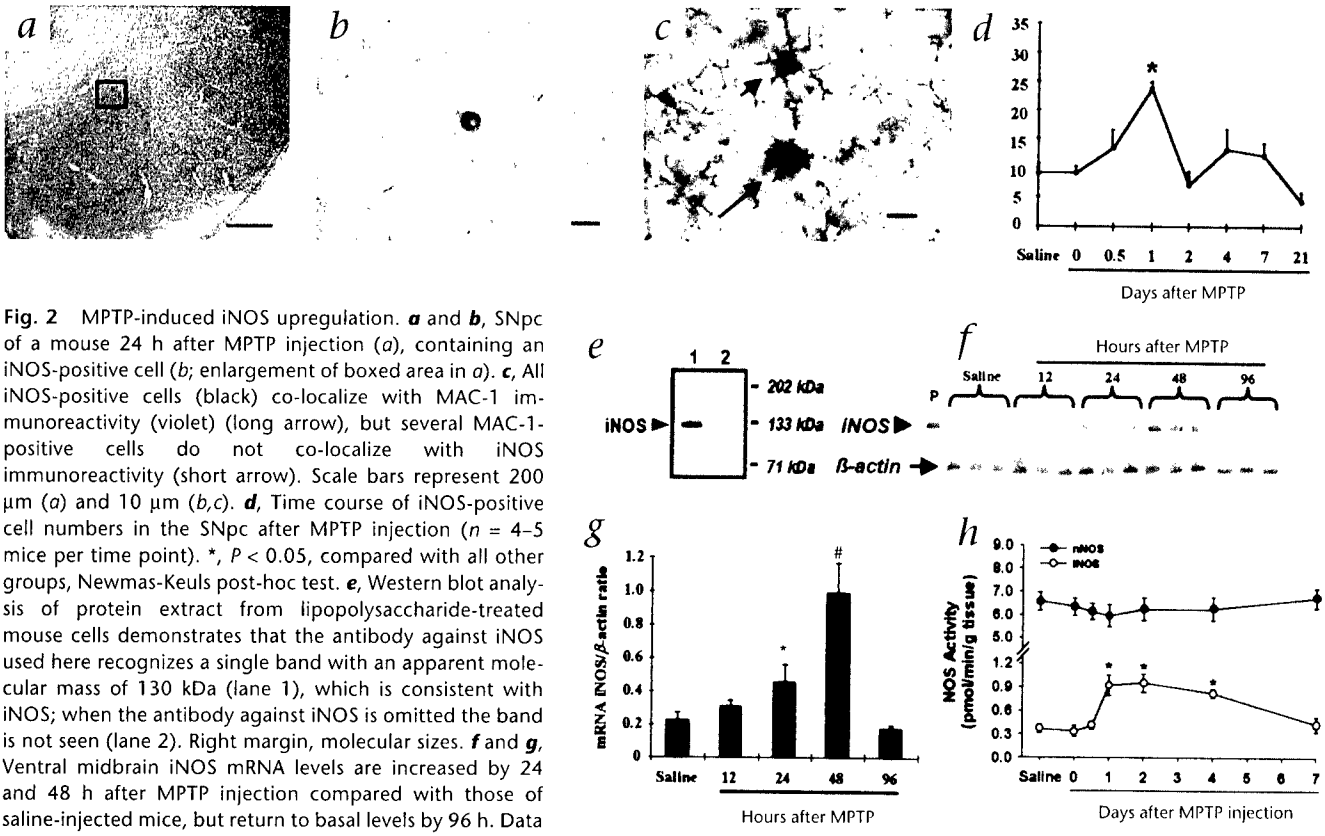

Fig. 2 MPTP-induced iNOS upregulation. ***a*** and ***b***, SNpc of a mouse 24 h after MPTP injection (*a*), containing an iNOS-positive cell (*b*; enlargement of boxed area in *a*). ***c***, All iNOS-positive cells (black) co-localize with MAC-1 immunoreactivity (violet) (long arrow), but several MAC-1-positive cells do not co-localize with iNOS immunoreactivity (short arrow). Scale bars represent 200 μm (*a*) and 10 μm (*b,c*). ***d***, Time course of iNOS-positive cell numbers in the SNpc after MPTP injection (n = 4–5 mice per time point). *, $P < 0.05$, compared with all other groups, Newmas-Keuls post-hoc test. ***e***, Western blot analysis of protein extract from lipopolysaccharide-treated mouse cells demonstrates that the antibody against iNOS used here recognizes a single band with an apparent molecular mass of 130 kDa (lane 1), which is consistent with iNOS; when the antibody against iNOS is omitted the band is not seen (lane 2). Right margin, molecular sizes. ***f*** and ***g***, Ventral midbrain iNOS mRNA levels are increased by 24 and 48 h after MPTP injection compared with those of saline-injected mice, but return to basal levels by 96 h. Data are from three mice per group and are representative of at least three independent experiments. P, iNOS-positive control (lipopolysaccharide-treated mouse cells). *, $P < 0.05$ and #, $P < 0.01$, higher than all other groups, Newmas-Keuls post-hoc test. ***h***, Ventral midbrain iNOS activity is significantly increased 1 d after MPTP injection, is still increased at 4 d although it returns to basal levels by 7 d after MPTP injection, whereas ventral midbrain nNOS activity remains unchanged throughout. *$P < 0.01$, higher than all other groups, Newmas-Keuls post-hoc test.

in SNpc microglia/macrophages[23]. However, our study provides essential insights into these autopsy findings, by indicating that iNOS upregulation is not due to the chronic use of anti-PD drugs such as L-dopa, nor is it an alteration that occurs at the very end of the disease process. Instead, our observation of increased iNOS immunoreactivity and enzymatic activity in MPTP-injected mice (Fig. 2) fits with the idea that iNOS-mediated NO and superoxide production[32] may contribute to the neurodegenerative process in this model and in PD. However, NO is membrane-permeable and can diffuse to neighboring neurons, whereas superoxide cannot readily transverse cellular membranes[39], making it unlikely for microglial-derived extracellular superoxide to gain access to dopaminergic neurons and directly trigger intracellular toxic events. Alternatively, NO could react with superoxide in the extracellular space to form the very reactive tissue-damaging species, peroxynitrite, which can cross the cell membrane and injure neurons. Therefore, microglial-derived superoxide, by contributing to peroxynitrite formation, may be important in this model. The presumed absence of direct involvement of extracellular superoxide in MPTP neurotoxic process, however, does not contradict the instrumental role of intracellular superoxide in this model[6], especially that produced within dopaminergic neurons consequent to the mitochondrial electron transport chain blockade by MPP^+.

Consistent with the involvement of iNOS in the MPTP neurotoxic process is our demonstration that approximately twice as many SNpc dopaminergic neurons survived in *iNOS*$^{-/-}$ mice as in their wild-type littermates after MPTP injection (Table 1). As activated microglia can also exert deleterious effects unrelated to NO, it must be emphasized that *iNOS*$^{-/-}$ mice showed no evidence of impaired microglial activation in response to MPTP (Fig. 3) and *iNOS*$^{-/-}$ macrophages, which do not produce NO, remain responsive to interferon-γ and, once activated, preserve their respiratory burst capacity[40] that includes the formation of superoxide[41]. Moreover, ablation of iNOS was not associated with alterations in the formation of the MPTP active metabolite MPP^+ (Table 3), which is the most important modulating factor of MPTP potency[26]. Given these data, the resistance of *iNOS*$^{-/-}$ mice to MPTP may result from the lack of iNOS expression and the consequent reduced NO formation, and not from either a microglial-deficient respiratory burst capacity or an altered MPTP metabolism. Unexpectedly, the resistance of the SNpc dopaminergic neurons in *iNOS*$^{-/-}$ mice was not accompanied by a similar sparing of striatal dopaminergic fibers, given that the levels of dopamine and metabolites after MPTP injection were similarly decreased in *iNOS*$^{-/-}$ mice and their wild-type littermates (Table 2). In the SNpc, MPTP caused significant upregulation of iNOS and, constitutively, there are only a few midbrain nNOS-positive neuronal elements that are not of dopaminergic nature and that do not have a close relationship with SNpc dopaminergic neurons[9,42]. In contrast, in the striatum, MPTP did not cause any detectable iNOS upregulation and, constitutively, there are many nNOS-positive neu-

rons and nerve fibers[9]. Therefore, the substantial preservation of the SNpc dopaminergic neurons coinciding with the loss of striatal dopaminergic fibers in MPTP-injected *iNOS*$^{-/-}$ mice can be explained by the fact that MPTP-mediated damage of striatal dopaminergic nerve fibers, unlike that of SNpc dopaminergic neurons, does not rely much on NO produced by iNOS, but instead on other NOS isoforms such as nNOS (ref. 20).

Peak ventral midbrain iNOS enzymatic activity was about 15% that of nNOS enzymatic activity (Fig. 2), raising the question of how such a non-predominant component of total NOS enzymatic activity could substantially modulate MPTP-induced SNpc injury. The answer may reside in the distance that NO must travel from the site of release to the dopaminergic neurons. Indeed, as described above, ventral midbrain nNOS-positive neuronal elements are located far from dopaminergic structures and thus it is likely that the amount of nNOS-derived NO that succeeds in reaching the target neurons is much less than could be expected, given the nNOS catalytic activity. In contrast, ventral midbrain iNOS-positive microglial cells are in close proximity to dopaminergic structures and thus the amount of iNOS-derived NO that succeeds in reaching the target neurons is conceivably very substantial. Also relevant is the fact that nNOS activity is under the dynamic regulation of calcium, thus it is probable that midbrain nNOS does not produce anywhere near 600% as much NO as iNOS.

Table 4 Nitrotyrosine levels (ng/μg protein)

	Frontal Cortex	Striatum	Ventral Midbrain	Cerebellum
Wild-type				
Saline	18.9 ± 3.7	30.9 ± 2.1	13.3 ± 3.2	21.6 ± 3.2
MPTP	19.1 ± 2.1	67.8 ± 3.1*	31.7 ± 1.7*	23.5 ± 2.2
iNOS$^{-/-}$				
Saline	18.4 ± 3.5	28.3 ± 2.5	14.0 ± 2.5	20.6 ± 1.8
MPTP	19.6 ± 2.5	68.4 ± 2.9**	21.9 ± 1.7***	19.1 ± 2.0

*, $P < 0.01$, higher than saline-injected mice; **, $P < 0.01$, higher than saline-injected mice but not MPTP-injected wild-type mice; ***, $P < 0.05$, higher than saline-injected mice but lower than MPTP-injected wild-type mice; Newman-Keuls post-hoc test. Data represent mean ± s.e.m. for three to four mice per group and treatment.

As for mechanisms, NO is a weak oxidant and thus it is not, by itself, sufficiently damaging to participate directly in MPTP deleterious effects. Alternatively, NO-derived species with stronger oxidant properties, such as peroxynitrite, can unquestionably cause direct injury to the dopaminergic neurons[43]. In addition, peroxynitrite can nitrate tyrosine residues[44], which may serve as a stable biomarker for peroxynitrite actions. Supporting the possibility of involvement of peroxynitrite in MPTP neurotoxic process is our demonstration that nitrotyrosine levels were significantly increased after MPTP injection only in brain regions known to be susceptible to the toxin (Table 4), which is consistent with previous studies[19,22,45]. Even more compelling, MPTP-injected *iNOS*$^{-/-}$ mice, which showed significantly less SNpc dopaminergic neuronal loss (Table 1), also showed significantly smaller increases in ventral midbrain nitrotyrosine levels than their wild-type counterparts (Table 4).

Our data provide evidence for a pivotal role for microglial iNOS-derived NO in the cascade of deleterious events that ultimately leads to SNpc dopaminergic neuronal death in the MPTP mouse model and in PD. Therefore, our study indicates that inhibition of iNOS may be a valuable target for the development of new therapies for PD aimed at attenuating the actual loss of dopaminergic neurons. However, this study also shows that iNOS inhibition may not be efficacious in preserving striatal nerve fibers from MPTP neurotoxicity. This indicates that the ideal therapeutic approach for PD may require the combination of iNOS inhibitors with other agents that have strong abilities in promoting nerve fiber re-growth and in stimulating dopaminergic function as well as in preserving dopaminergic nerve terminals. So far, multi-drug strategies have proved to be successful in fitting other pathological conditions, such as HIV infection and cancer.

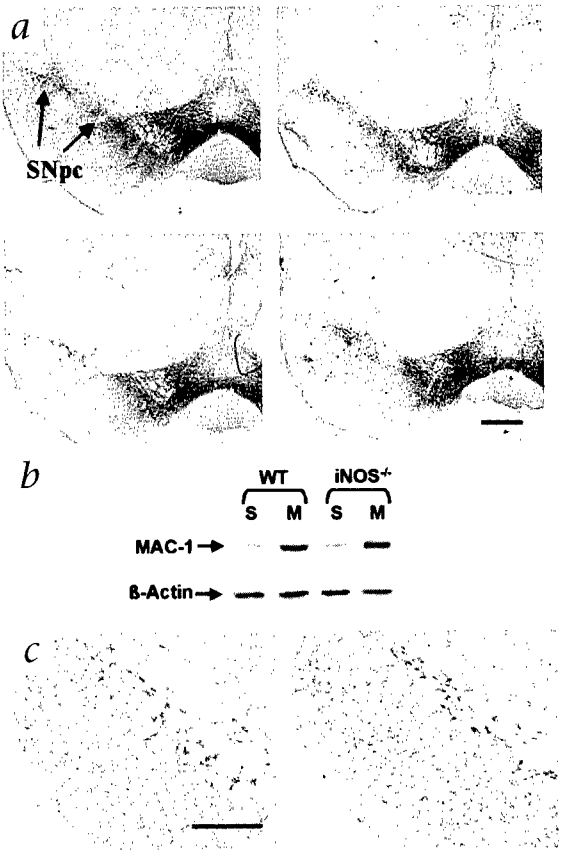

Fig. 3 MPTP-induced neuronal loss and microglial reaction in *iNOS*$^{-/-}$ mice. ***a***, SNpc TH-positive neurons are twice as resistant to MPTP in *iNOS*$^{-/-}$ mice than in wild-type (WT) littermates, 7 d after MPTP injection. In addition, there is no noticeable difference in TH-positive neuron density after saline injection between *iNOS*$^{-/-}$ mice and WT littermates (Table 1, actual neuronal counts). ***b*** and ***c***, Ventral midbrain western blot analysis (*b*) and SNpc immunohistochemical analysis (*c*) for MAC-1, showing the similarity of the microglial response between the *iNOS*$^{-/-}$ mice and their wild-type littermates (WT), 24 h after MPTP injection. Scale bar represents 200 μm (*a* and *c*, shown in *c*).

Methods

Animals and treatment. Eight-week-old male C57/bl mice (Charles River Laboratories, Wilmington, Massachusetts) and iNOS-deficient mice (C57/bl-NOS2^{tm1Lau}; Jackson Laboratories, Bar Harbor, Maine) and their wild-type littermates were used. Mice (n = 4–6 per group) received four intraperitoneal injections of MPTP-HCl (20 mg/kg of free base; Research Biochemicals, Natick, Masssachusetts) in saline at 2-hour intervals in 1 day, and were killed at selected times 0–21 days after the last injection. Control mice received saline only. This protocol was in accordance with the NIH guidelines for use of live animals and was approved by the Institutional Animal Care and Use Committee of Columbia University and Johns Hopkins University School of Medicine.

MAC-1, GFAP, and iNOS immunohistochemistry. These were done as described[46] on cryostat-cut sections (30 μm in thickness) encompassing the entire midbrain, and used the following primary antibodies (at the following dilutions): rat antibody against MAC-1 (1:1,000 ; Serotec, Raleigh, North Carolina), rabbit antibody against GFAP (1:1,000; Dako, Carpinteria, California), and rabbit antibody against iNOS (1:1,000; Transduction Laboratories, Lexington, Kentucky). The double-immunostaining procedure for iNOS and MAC-1 or GFAP was used as described[47] with minor modifications. Because the number of iNOS-positive cells in the SNpc at any given time was very small, we could not use a stereological method[48], but instead have used an assumption-based method[20], following strict guidelines[49] to ensure the validity of our quantification technique.

RNA extraction and RT–PCR for iNOS and β–actin. These were done using methods described[46], using the same pair of primers for iNOS and β-actin, PCR conditions, and a slightly modified reaction mixture (20 μl) consisting of 1 μl cDNA template, 18 μl Supermix (Life Technologies), 10 fmol ^{32}P-dCTP (specific activity, 3,000 Ci/mmol; NEN) and 4–10 pmol of each specific primer. After amplification, the products were separated by 5% polyacrylamide gel electrophoresis. After being dried, gels were exposed to phosphoimager screens (BioRad, Hercules, California), and optical densities were determined using a computerized image analysis system (BioRad, Hercules, California).

Assay of iNOS catalytic activity. nNOS and iNOS catalytic activities were assayed in midbrain samples from four mice per time point and condition, by measuring both the calcium-dependent and calcium-independent conversion of ^{3}H-arginine to ^{3}H-citrulline as described[20,46]. No substantial ^{3}H-citrulline production occurred in the absence of nicotine adenine dinucleotide phosphate, and this represented background counts.

Measurement of striatal dopamine, DOPAC and HVA levels. High-performance liquid chromatography (HPLC) with electrochemical detection was used to measure striatal levels of dopamine, DOPAC and HVA using a method that has been described[20], with minor modifications of the mobile phase. At 7 d after the last MPTP injection, *iNOS*$^{-/-}$ mice and wild-type littermates (four to six per group) were killed, and the striata were dissected out and processed for HPLC measurement. The modified mobile phase consisted of 0.15 M monochloroacetic acid, pH 3.0, 200 mg/l sodium octyl sulfate, 0.1 mM EDTA, 4% acetonitrile and 2.5% tetrahydrofuran.

Measurement of striatal MPP^{+} levels. HPLC with ultraviolet detection (wavelength, 295 nm) was used to measure striatal MPP^{+} levels using a method that has been described[20]. Groups of *iNOS*$^{-/-}$ mice and wild-type littermates (four per time point) were killed at 90, 120, 180 and 360 min after the fourth intraperitoneal injection of 20 mg/kg MPTP, and the striata were dissected out and processed for HPLC measurement as described[20].

***iNOS*$^{-/-}$ TH and Nissl staining and stereology.** The total number of TH- and Nissl-stained SNpc neurons were counted in five mice per group using the optical fractionator method as described[48]; this is an unbiased method of cell counting that is not affected by either the volume of reference (SNpc) or the size of the counted elements (neurons)[50]. TH immunostaining was done as described[20], using an affinity-purified polyclonal antibody against TH (1:2,000 dilution; Calbiochem, San Diego, California).

Immunoblots. Mouse brain protein extracts from selected regions were prepared as described[45]. For western blot analysis, 10% SDS–PAGE and transfer of proteins to nitrocellulose membrane were done as described[45], and blots were probed with either antibody against MAC-1 (1:1,000 dilution; Serotec, Raleigh, North Carolina), antibody against GFAP (1:2,000 dilution; DAKO), antibody against iNOS (1:1,000 dilution; Transduction Laboratories, Lexington, Kentucky), or antibody against β-actin (1:5,000 dilution; Sigma). For dot–blot analyses, 100 μg of protein extracts were loaded onto the nitrocellulose membrane, and blots were probed with an affinity-purified polyclonal antibody against nitrotyrosine (1:1000 dilution; a gift from H. Ischiropoulos). For all blots, bound primary antibody was detected using a horseradish-conjugated antibody against IgG (1:2,000 dilution; Amersham) and a chemiluminescent substrate (SuperSignal Ultra; Pierce Chemical, Rockford, Illinois). X-ray films (Kodak BioMax MS) were scanned on a HP-4C Scanjet and bands were quantified using NIH-Image 1.62 software. For all dot–blot analyses, optical densities were converted into nanograms of nitrotyrosine using a standard curve generated from different concentrations of nitrated bovine serum albumin (a gift from H. Ischiropoulos) corresponding to 14–350 μg nitrated protein or 0.4–10 ng nitrotyrosine. In preliminary experiments, we confirmed the specificity of nitrotyrosine immunoreactivity by demonstrating that immunostaining is abolished either by co-incubating the antibody against nitrotyrosine with 10 mM nitrotyrosine or by treating blots with 10 mM sodium hydrosulfite, to reduce nitrotyrosine to aminotyrosine, before incubation with the primary antibody[51].

Statistical analysis. All values are expressed as the mean ± s.e.m. Differences among means were analyzed using one- or two-way ANOVA with time, treatment or genotype as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Newman-Keuls post-hoc testing. In all analysis, the null hypothesis was rejected at the 0.05 level.

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